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Ovarian Hormone-Induced Neural Plasticity in the Hypothalamic Ventromedial Nucleus

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Abstract
Ovarian hormones act throughout the brain and body to influence a range of functions and behaviors. Estrogens and progesterone are neuroprotective and have important health implications. However, our understanding of the mechanisms underlying many of their actions is incomplete. The goal of this thesis is to elucidate details of ovarian hormone-induced neural plasticity, specifically as it relates to female reproductive behavior and dendritic morphology in the hypothalamic ventromedial nucleus (VMH). First, I tested the hypothesis that the mechanisms of female receptivity are conserved across two species with very different mating strategies: prairie voles, which exhibit monogamy, induced estrous, and induced ovulation, and rats, which do not. Utilizing Golgi impregnation, I demonstrated that, as in rats, dendrite length in the VMH of female prairie voles is correlated with mating status, which in turn is associated with estradiol concentration. Next, I tested the hypothesis that oxytocin, a neuromodulator that promotes mating behavior, is a marker for estradiol-induced synaptic reorganization in the VMH. Using immunoelectron microscopy, I revealed that estradiol reduces the presence of dendrites that extend out into the adjacent lateral fiber plexus and increases the innervation of the remaining dendrites, which have internalized oxytocin. Finally, I tested the hypothesis that estradiol has short-term effects on proteins associated with synaptic reorganization, namely the actin-associated protein cofilin and ionotropic AMPA glutamate receptor subunits GluA1 and GluA2 in the VMH. Using immunohistochemistry, I discovered that estradiol rapidly alters phosphorylation of cofilin and the levels of GluA1. Together, these findings demonstrate that estradiol actions in the VMH are evolutionarily conserved, affect oxytocin synapses, and rapidly alter regulation of actin polymerization and glutamate signaling.

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OVARIAN HORMONE-INDUCED NEURAL PLASTICITY IN THE HYPOTHALAMIC VENTROMEDIAL NUCLEUS

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ABSTRACT

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Loretta M. Flanagan-Cato

Ovarian hormones act throughout the brain and body to influence a range of functions and behaviors. Estrogens and progesterone are neuroprotective and have important health implications. However, our understanding of the mechanisms underlying many of their actions is incomplete. The goal of this thesis is to elucidate details of ovarian hormone-induced neural plasticity, specifically as it relates to female reproductive behavior and dendritic morphology in the hypothalamic ventromedial nucleus (VMH). First, I tested the hypothesis that the mechanisms of female receptivity are conserved across two species with very different mating strategies: prairie voles, which exhibit monogamy, induced estrous, and induced ovulation, and rats, which do not. Utilizing Golgi impregnation, I demonstrated that, as in rats, dendrite length in the VMH of female prairie voles is correlated with mating status, which in turn is associated with estradiol concentration. Next, I tested the hypothesis that oxytocin, a neuromodulator that promotes mating behavior, is a marker for estradiol-induced synaptic reorganization in the VMH. Using immunoelectron microscopy, I revealed that estradiol reduces the presence of dendrites that extend out into the adjacent lateral fiber plexus and increases the innervation of the remaining dendrites, which have internalized oxytocin. Finally, I tested the hypothesis that estradiol has short-term effects on proteins associated with synaptic reorganization, namely the actin-associated protein cofilin and ionotropic AMPA glutamate receptor subunits GluA1 and GluA2 in the VMH. Using immunohistochemistry, I discovered that estradiol rapidly alters phosphorylation of cofilin and the levels of GluA1. Together, these findings demonstrate that estradiol actions in the VMH are evolutionarily conserved, affect oxytocin synapses, and rapidly alter regulation of actin polymerization and glutamate signaling.

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CHAPTER 1: Introduction

The ovarian hormones estradiol and progesterone have been associated with a vast number of effects on both the body and the brain. One of the behaviors dependent upon hormone action in the brain is lordosis, the hallmark of female sexual receptivity in a number of species. As lordosis is an important component of reproduction that results in propagation of the species, it is a biologically significant behavior which has been well conserved evolutionarily. It is a simple reflexive posture, but is associated with a suite of changes in neurotransmitter, peptide, effector, and receptor actions, as well as structural transformations. Amazingly, this elaborate cascade of events occurs every four to five days across a rat's estrous cycle to control the timing of reproductive behavior and maximize likelihood of impregnation. The ovarian hormone-induced changes exhibit a complex spatial and temporal specificity, and while it is a well studied phenomenon, especially in rats, many mechanistic details remain to be clarified.

The focus of this dissertation is to investigate ovarian hormone-dependent changes in dendritic morphology within the vlVMH, the brain area associated with female sexual behavior, by 1) extending ovarian hormone-associated dendrite analysis to another species with a different mating strategy; 2) studying the estradiol-induced synaptic reorganization of dendrites emanating from the vlVMH and the subcellular localization of oxytocin (OT), a peptide important for behavior, in that area; 3) examining the time course and ovarian hormone regulation of expression of AMPA receptor subunits GluA1 and GluA2 and the actin-associated protein cofilin related to estradiol-induced spinogenesis in the vlVMH.

Lordosis as a Model System

Lordosis is a sex-specific behavioral reflex in which a female assumes a rigid stance, arches her back, and exposes her genital region. This posture signals sexual receptivity to the male and usually occurs in response to flank and vaginal-cervical stimulation. Lordosis facilitates copulation, which increases the likelihood of impregnation, and as a result, the propagation of

genes. Therefore, lordosis is a biologically significant behavior. Furthermore, it is highly evolutionarily conserved, and is practiced by most mammals and even lizards capable of asexual reproduction (Grassman and Crews, 1986; Flanagan-Cato, 2011).

The reliance of lordosis execution on ovarian hormones is well established in many mammals. Both estradiol and progesterone are necessary for the behavior to be executed fully, although there is modest lordosis in the presence of estradiol alone (Boling and Blandau, 1939). Indeed, female estrogen receptor-α knockout mice fail to exhibit lordosis (Ogawa et al., 1998). Progesterone in the absence of estradiol has no effect on lordosis behavior, as progesterone receptor expression is dependent upon the activation of estradiol receptors (Blaustein et al., 1988). Accordingly, ovariectomized (OVX) females fail to engage in this behavior. Subsequent steroid hormone replacement rescues lordosis in OVX females. Likewise, sexual receptivity fluctuates concurrently with estrous cycle in intact females, as ovarian hormones naturally rise and fall.

Lordosis is a valuable model system to study ovarian hormone action because it is predictable and reliable. Lordosis behavior is expressed only for several hours every four days over the natural estrous cycle or after a specific concentration of exogenous hormones (Boling and Blandau, 1939). The arched-back posture is robust, easy to recognize, and straightforward to quantify. Therefore, experimental manipulations are well suited to have salient and consistent effects on behavior and neural structure, establishing lordosis as an effective and useful system in which to study ovarian hormone-induced neural plasticity, the focus of this thesis. However, while lordosis behavior and a number of details of underlying mechanisms have been well described, several key questions remain, including some aspects of the neurochemistry, synaptic organization, and cell types involved.

VMH is required for lordosis expression

As the dependence of lordosis on estradiol and progesterone has been thoroughly demonstrated, so has the importance of the ventromedial nucleus of the hypothalamus (VMH) in the neurological-behavioral lordosis circuit. The involvement of this nucleus in sexual behavior is

highly conserved and is implicated in a variety of species, including non-human primates (see Flanagan-Cato, 2011; Aou et al., 1988). Additionally, the VMH exhibits sexual dimorphism, with males exhibiting a larger overall volume of the nucleus as well as larger neurons (Madeira et al., 2001; Dorner and Staudt, 1969; Matsumoto and Arai, 1983; Dugger et al., 2007; Griffin and Flanagan-Cato, 2009). Likewise, expression of steroid hormone and other receptor types exhibit sex differences, as do the actions of several hormones, neurotransmitters such as GABA and serotonin, and other molecules that modulate sex behavior (Grattan and Selmanoff, 1997; Willoughby and Blessing, 1987).

The VMH contains progesterone receptors and several types of estrogen receptors (Blaustein et al., 1988). Estrogen receptors alpha (ERα) and beta (ERβ) are found in the nucleus or on the membrane and are thus capable of genomic as well as rapid signaling actions (Pfaff and Keiner, 1973; Simerly et al., 1990; Don Carlos et al., 199; Green et al., 1986; Kuiper et al., 1996; Kelly and Ronnekleiv, 2012; discussed later in this chapter). Other membrane-associated ERs are currently being investigated, including GPR30 and a novel Gαq-coupled membrane ER (Gαq-mER) (Kelly and Ronnekleiv, 2012). Thus, estradiol action in this brain area is apparent.

The presence of these receptors coupled with evidence that estradiol delivered directly to the VMH effectively generates sexual receptivity, further substantiates the importance of ovarian hormone action in the VMH for lordosis induction (Dorner et al., 1968). Likewise, several lesion studies have demonstrated that damage to the area diminished lordotic response (Kennedy, 1964; Pfaff and Sakuma, 1979), as does the blockade of action potentials with local application of tetrodotoxin (Harlan et al., 1983). It eventually became clear that the female sexual behavior-promoting effects involving the VMH were specific to its ventrolateral subdivision (vlVMH); knife cuts affecting lateral-projecting neurons inhibited display of lordosis while damage to the posterior projections (dorsomedial VMH; dmVMH) had no effect (Manogue et al., 1980).

Neuronal activity in the VMH is also affected by ovarian hormones and mating cues. VMH neurons are responsive to flank and vaginal-cervical stimulation (VCS) (Bueno and Pfaff, 1976). Likewise, electrical stimulation of the VMH augments lordosis behavior (Pfaff and Sakuma,

1979). Finally, female sexual behavior induces expression of immediate early genes, indicative of recent neuronal activity (Flanagan et al., 1993; Tetel et al., 1994; Flanagan and McEwen, 1995; Pfaus et al., 1993; Wersinger et al., 1993; Rowe and Erskine, 1993; Polston and Erskine, 1995). These collective data strengthen the relationship between ovarian hormones and female sexual behavior and stress the relevance of the ventrolateral subdivision of the VMH to lordosis behavior.

As previously mentioned, the vlVMH receives little afferent input from other brain areas, but there are some connections with the medial amygdala (Ono et al., 1985). As for output, although the lordosis circuit has not been completely defined, it includes projections from the VMH to the periaqueductal gray, which projects to the medullary reticular formation, then the lumbar ventral horn, and finally the lumbar epaxial muscles, which are necessary for the lordosis posture (Pfaff and Scwartz-Giblin, 1988; Flanagan-Cato et al., 2001).

Cytoarchitecture of the VMH

The VMH is a nucleus adjacent to the third ventricle and dorsal to the median eminence and hypothalamic arcuate nucleus. It is divided into the ventrolateral and dorsomedial subdivisions (vl and dm, respectively), with the former associated with sexual behavior, as discussed above, and the latter with ingestive behavior (Millhouse, 1973; Flanagan-Cato, 2011). Besides these designations, the VMH has no definitive organization, but numerous studies have gathered information about receptor expression location, and projection patterns. Several cell types have emerged: ERα-expressing cells, non- ERα-expressing cells that have axons that project to the periaqueductal gray (PAG), non-ERα-expressing cells with short primary dendrites that exhibit estradiol-induced spinogenesis, and cells with long primary dendrites that change length in response to ovarian hormones (Calizo and Flanagan-Cato 2000; Calizo and Flanagan-Cato, 2002; Calizo and Flanagan-Cato, 2003; Griffin and Flanagan-Cato, 2008; Daniels and Flanagan-Cato, 2000; Auger et al., 1996). Figure 1.1 illustrates these vlVMH cell types. Currently, it is not known whether the cells with dendrites that exhibit hormone-induced length changes express ERα. Thus, they may overlap with one or more of the other cell types identified. Likewise, some of the other

preliminarily classified non-ERα-expressing cells may overlap as well. For example, cells with axons that project to the PAG may have long primary dendrites that change length after hormone treatment or have short primary dendrites that undergo an estradiol-induced increase in spine density. Finally, some of the PAG-projecting neurons (Pjn cells) and some of the ERα-expressing cells were activated in response to mating, as indicated by expression of the immediate early gene Fos. Approximately 41% of Fos+ cells neither contained ER alpha nor projected to the PAG (Calizo and Flanagan-Cato, 2003). Additional cell types likely exist, but these classifications may provide insight into VMH connections and mechanisms.

Additional colocalization of receptors, proteins, and effectors has also been characterized to some degree. Profiles of mRNA in single VMH cells uncover a high colocalization of ERα, OTR, and PKC (Devidze et al., 2005). ERα cells also express substance P and are largely glutamatergic (Daniels and Flanagan-Cato, 2003; Eyigor et al., 2004). It has not yet been elucidated where membrane ERs are expressed but it will be a future goal to integrate the existing data to develop a full characterization of the cell types in the VMH.

While cell types are not yet thoroughly defined, the general dendritic arbor of vlVMH neurons is well described (see Figure 1.2). Each neuron has one very long dendrite emanating from the soma termed the long primary dendrite, most of which extend in the ventrolateral direction, toward the adjacent lateral fiber complex (LFC). In addition, neurons have an average of two short primary dendrites that also extend directly from the cell body, but which are shorter than the long primary dendrite and do not exhibit directional preference. Approximately one to two secondary dendrites usually branch off of primary dendrites. These distinct classifications of dendrites often exhibit differential structural changes in response to ovarian hormones, an effect that will be discussed in detail in subsequent chapters (Calizo and Flanagan-Cato, 2000; Calizo and Flanagan-Cato, 2002; Griffin and Flanagan-Cato, 2008).

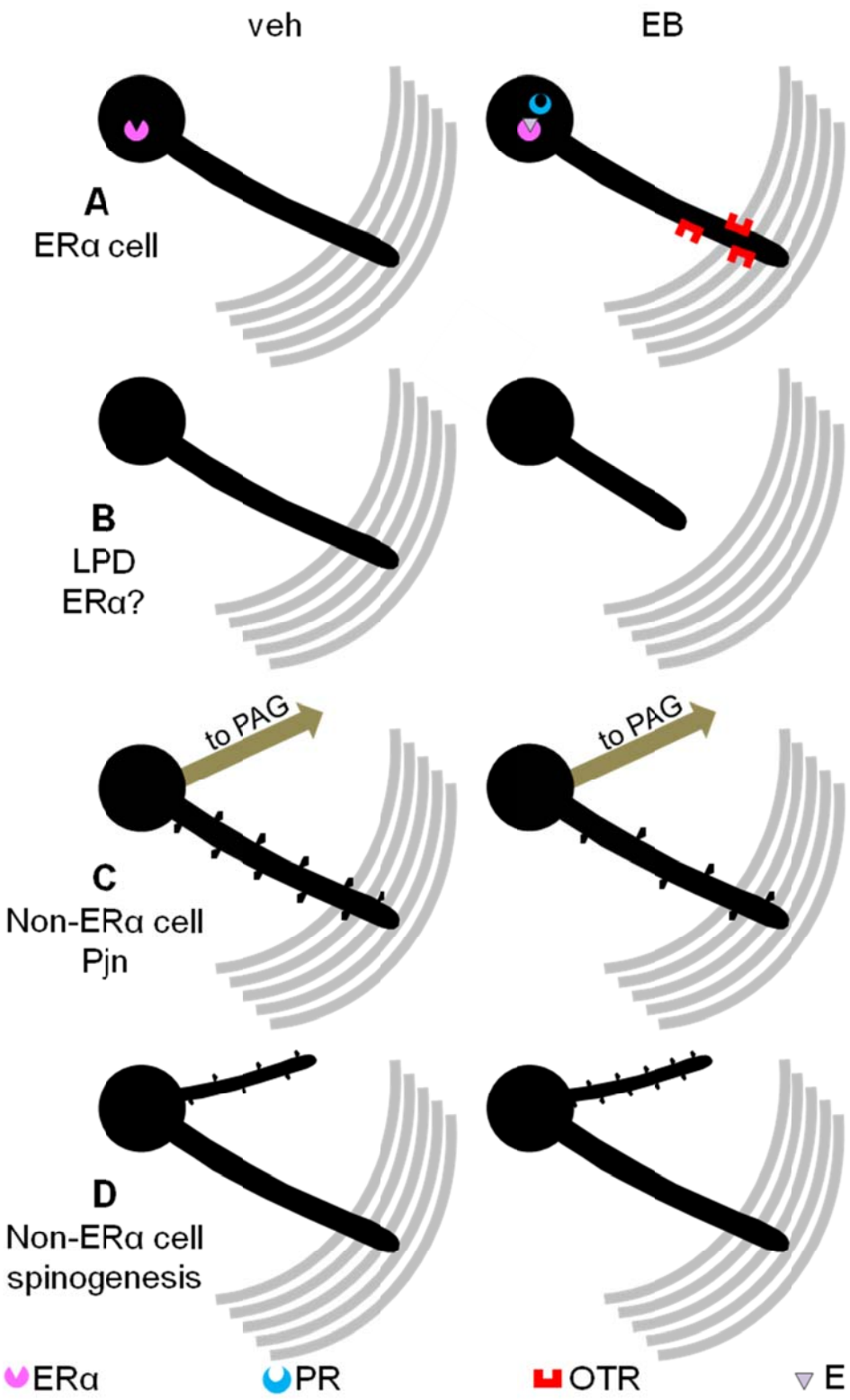

Figure 1.1. Proposed cell types in the vlVMH.

A. Progesterone receptors and oxytocin receptors (OTR) are upregulated by estradiol treatment in ERα-expressing cells, which do not exhibit estradiol-induced changes in long primary or short primary dendrites (Blaustein et al., 1988; de Kloet et al., 1986; Calizo and Flanagan-Cato, 2000; Calizo and Flanagan-Cato, 2002). B. Some vlVMH neurons have long primary dendrites that retract in response to estradiol treatment (Griffin and Flanagan-Cato, 2008). It is not clear whether these cells express ERα. C. Pjn neurons have axons that project to the periaqueductal gray (PAG) and long primary dendrites exhibit an estradiol-induced decrease in spine density. These cells do not express ERα (Calizo and Flanagan-Cato, 2002). D. A subset of non-ERα-expressing cells undergo estradiol-induced spinogenesis on short primary dendrites (Calizo and Flanagan-Cato, 2002). vlVMH neurons are shown in black; gray lines represent axons in the lateral fiber complex (LFC); beige arrows represent axons projecting to the periaqueductal gray (PAG); ERα = estrogen receptor alpha; PR = progesterone receptor; OTR – oxytocin receptor; E = estradiol. Adapted from Flanagan-Cato, 2011.

Finally, an ultrastructural study revealed that approximately one third of synapses observed in the vlVMH are axospinous and excitatory. Of the numerous axodendritic synapses, only 10-15% appeared to be inhibitory (Nishizuka and Pfaff, 1989). Interestingly, the human VMH seems to exhibit similar neuroanatomy, expressing estrogen and oxytocin receptors, as well as similar afferent inputs and connections (see Flanagan-Cato, 2011 for review). Thus, while the vlVMH is not clearly mapped in terms of cell types and their connections, various lines of research have provided valuable descriptions of the cells in this area.

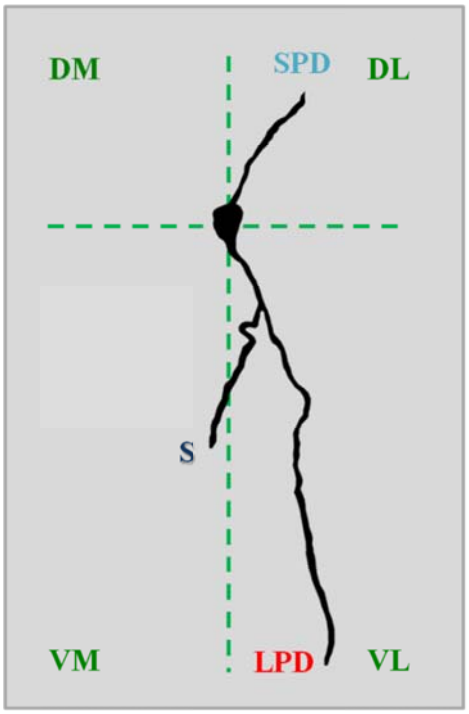

Figure 1.2. Representative vlVMH neuron.

Each neuron has one very long dendrite emanating from the soma termed the long primary dendrite, labeled "LPD" in red. Most LPDs extend in the ventrolateral direction, toward the adjacent lateral fiber complex (LFC). Green dotted lines distinguish the four possible directions of dendrite extension: DM, dorsomedial; DL, dorsolateral; VM, ventromedial; VL, ventrolateral. vlVMH neurons have an average of two short primary dendrites that also extend directly from the cell body, but which are shorter than LPDs. A short primary dendrites are indicated here by a blue "SPD," and SPDs do not exhibit directional preference. Approximately one to two secondary dendrites, indicated by a purple "S," usually branch off of primary dendrites and do not exhibit directional preference.

Surrounding the cell-dense vlVMH is a fiber field, the lateral fiber complex (LFC), which contains few cell bodies but is comprised of axonal processes *en route* to the median eminence. Early literature noted that these fibers do not project into the vlVMH, but dendrites from the nucleus appeared to ramify out into the LFC (see Figure 1.1) (Millhouse, 1973). This and subsequent observations described a juxtaposition of the vlVMH dendrites and LFC axons, indicating possible innervation of the former by the latter (Daniels and Flanagan-Cato, 2000). Further suggesting the importance of possible connections, severing fibers in the LFC region results in a decrease in female sexual behavior (Nishizuka and Pfaff, 1989). However, it was a recent ultrastructural study performed in our lab that confirmed synaptic connections between the LFC and vlVMH for the first time (Griffin et al., 2010). The presence of vlVMH-LFC synapses may allow for effective delivery of neurochemicals important for behavior. For example, gonadotropin releasing hormone, norepinephrine, oxytocin, and serotonin are all present in the LFC and modulate lordosis (Merchenthaler et al., 1984; Swanson and Hartman, 1975; Schumacher et al., 1989). These neuromodulators are discussed in detail later in this Introduction. Additionally, Chapter Three includes an extension of the main finding of synaptic connections between the vlVMH and LFC by analyzing synaptic reorganization in this area.

Neuroplasticity in the VMH

An important feature of female sexual receptivity is neuroplasticity, which is dependent upon ovarian hormones. Estradiol and progesterone treatment have various effects on VMH ultrastructure which likely contribute to its regulation of lordosis response. In the VMH, estradiol increases rough surfaced endoplasmic reticulum, condensation of nucleolar material, enlarged Golgi, and presence of pleiomorphic mitochondria. Additionally, neuron somata and nuclei are enlarged. These changes are indicative of metabolic stimulation. Axodendritic and axospinous synapses are also more abundant after estradiol treatment (Carrer and Aoki, 1982; Nishizuka and Pfaff, 1989; Frankfurt and McEwen, 1991). Estradiol and estradiol plus progesterone decrease the

number of secondary dendrites branching from long primary dendrites as well (Griffin and Flanagan-Cato, 2008).

Ovarian hormones have effects on dendritic length as well. In rats, intact females have longer total vlVMH dendrite lengths in proestrus, when lordosis behavior occurs, versus in diestrus, when ovarian hormone levels are low (Madeira et al., 2001). In ovariectomized (OVX) female rats, estradiol decreases the length of long primary dendrites (LPDs) extending from the vlVMH into the lateral fiber complex (LFC), while the subsequent addition of progesterone causes an extension of these dendrites within four hours (Griffin and Flanagan-Cato, 2008). OVX Syrian hamsters exhibit an increase in total VMH dendrite length in response to estradiol and estradiol plus progesterone (Meisel and Luttrell, 1990). The purpose of dendrite length changes may be to regulate connections that provide afferent input that drives behavior (see Figure 1.1B). Mechanisms of dendrite length changes, especially those that occur during adulthood, are not well described. However, some possibilities are explored in Chapter Five.

As hormone effects of vlVMH dendrite length appear to be functionally significant for lordosis behavior in rats, Chapter Two addresses possible conservation of arborization. Here, we investigated the effects of hormones on dendritic arbor in female prairie voles, another species that exhibits lordosis but a rare mating system that includes induced estrous, induced ovulation, and monogamy. We hypothesized that dendrite length correlates with mating status in female prairie voles, as it does in female rats. Prairie voles are further discussed later in this Introduction. Chapter Three then explores synaptic reorganization in the LFC as a result of hormone-induced extension and retraction of vlVMH long primary dendrite length in female rats, testing the hypothesis that estradiol and progesterone affect number of synaptic inputs on vlVMH dendrites.

Manipulations of estradiol and progesterone also affect dendritic spine density. Spinogenesis was observed with exogenous hormone administration as well as during the proestrus phase of intact females, when estradiol and progesterone are at their peak (Frankfurt et al., 1990; Madeira et al., 2001; Calizo and Flanagan-Cato, 2000). Specifically, estradiol causes an increase in spine density on short primary dendrites of non-ERα expressing vlVMH cells and a

decrease in spine density on long primary dendrites of non-ERα expressing vlVMH cells with axonal projections to the periaqueductal gray (PAG) (Calizo and Flanagan-Cato, 2000; Calizo and Flanagan-Cato, 2002; see Fig. 1.1C and D). It has not yet been elucidated whether these changes on different dendrite types are occurring on separate subsets of non-ERα expressing cells or they are occurring on the same cell type. These complex changes likely enable coordination of input and output in order to regulate the timing of reproductive behavior. In Chapter Four, I investigate the effects of ovarian hormones on proteins associated with spinogenesis at different timepoints. Specifically, the actin-associated protein cofilin and the ionotropic glutamate AMPAR subunits GluA1 and GluA2 are examined.

Neurotransmitters and neuromodulators important for lordosis

A number of other neurochemical contributors besides estradiol and progesterone have been demonstrated to be important in the VMH to promote lordosis behavior. Gamma-aminobutyric acid (GABA) immunoreactivity is present in the vlVMH, as are GABA-A receptors (Commons et al., 1999; Grattan and Selmanoff, 1997). GABAergic activity in the area has also been documented via spontaneous inhibitory postsynaptic currents (Jang et al., 2001). However, while GABA biosynthetic enzyme glutamic acid decarboxylase-65 (GAD-65) terminals are present in the VMH, neither GAD65 nor GAD67 are produced within the nucleus, suggesting a mechanism of action involving afferent input from other brain regions (Okamura et al., 1990; Patisaul et al., 2008). Additionally, only 10-15% of axodendritic synapses appear to be inhibitory (Nishizuka and Pfaff, 1989). GABA and GABA-A receptor agonists in the VMH promote sexual behavior (McCarthy et al., 1990) while GABA-A receptor antagonists diminish lordosis response (Roy et al., 1985).

Glutamate, α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or *N*-Methyl-D-aspartate (NMDA) delivered intracerebroventricularly or directly to the vlVMH attenuate lordosis, and AMPA and NMDA receptor antagonists facilitate sexual receptivity (Kow et al., 1985; McCarthy et al. 1991; Georgescu and Pfaus, 2006a; Georgescu and Pfaus, 2006b; Fleischmann et al., 1991). Both ionotropic and metabotropic glutamate receptors are expressed in the VMH, with NMDA

receptors being most abundant, followed by metabotropic receptors, kainate receptors, and AMPA receptors (Grattan and Selmanoff, 1997; Meeker et al., 1994). The source of glutamate in these inhibitory mechanisms may be ERα-expressing cells. As previously mentioned, 81% of neurons that express ERα mRNA coexpress vesicular glutamate transporter (VGLUT2) mRNA (Eyigor et al., 2004). Chapter Four of this thesis addresses AMPAR subunit expression over time in response to ovarian hormone treatment.

In addition to the main neurotransmitters, various neuromodulators have been implicated in the lordosis circuit. Within the vlVMH, the peptides enkephalin, substance P, and prolactin promote lordosis behavior (Nicot et al., 1997; Akesson and Micevych, 1988; Dornan et al., 1987; Dornan et al., 1990; Harlan et al., 1983). Interestingly, enkephalin and substance P are colocalized with ERs, and their levels are increased in response to estradiol treatment (Olster and Blaustein, 1990; Turcotte and Blaustein, 1997; Micevych et al., 1988; Romano et al., 1988). Similarly, neuronal nitric oxide synthase facilitates lordosis, is colocalized with ERα in the vlVMH, and is modulated by estradiol (Chu and Etgen, 1999; Rachman et al., 1998). Norepinephrine, acetylcholine, gonadotropin releasing hormone, and arginine-vasopressin (AVP) all also increase lordosis response via intracerebroventricular administration in hormone-primed OVX females (Kow et al., 1988; Vincent and Etgen, 1993). On the other hand, serotonin and cholecystokinin both attenuate female sexual behavior (Zemlan et al., 1973; Kow et al., 1988; Babcock et al., 1988; Akesson and Micevych, 1988). Finally, oxytocin modulates lordosis behavior, and is discussed below, and further in Chapter Three.

The role of oxytocin in lordosis

Oxytocin (OT) is a nonapeptide that is evolutionarily conserved across phyla to such an extent that virtually all vertebrate species possess an OT-like peptide (Gimpl and Fahrenholz, 2001). Many studies have established the role of OT in social contexts, including maternal behavior, pair-bonding/romantic attachment, peer association, social learning, memory, and anxiety. It has been linked to human trust, immune function, pain perception, and ingestive

behavior as well (see Gimpl and Fahrenholz, 2001; Lee et al., 2009; Ross and Young, 2009 for review).

Oxytocin receptor (OTR) expression is abundant in the brain, including throughout the olfactory system, several cortical areas, basal ganglia, limbic system, thalamus, hypothalamus, and brainstem (Gimpl and Fahrenholz, 2001). Despite this ubiquity, OT itself is produced in very specific brain areas. The peptide is synthesized primarily in magnocellular hypothalamic neurons of the paraventricular (PVN) and supraoptic (SON) nuclei, and to a lesser extent in parvocellular neurons of the PVN. From these nuclei, most OT is relayed to the pituitary and released into the bloodstream (Kelly and Ronnekleiv, 2012). However, small amounts of OT are also produced in the bed nucleus of the stria terminalis, medial preoptic area, and lateral amygdala.

Because of the disproportionate distribution of OT neurons relative to OTR neurons, the mechanism by which certain brain areas receives OT to regulate specific behaviors is not well characterized. A long-standing hypothesis postulates that magnocellular OT neurons project exclusively to the posterior pituitary while parvocellular neurons project to various regions within the brain. However, some researchers have suggested that centrally projecting OT axons may arise from the magnocellular neurons that project to the posterior pituitary as axon collaterals of the neurohypophyseal OT neurons. These collaterals may then release OT nonsynaptically or may innervate specific nuclei (reviewed in Ross et al., 2009). Evidence for this mechanism is suggested by numerous OT-immunoreactive fibers throughout the brain as well as the discovery of oxytocinergic synaptic connections in the nucleus accumbens and LFC (Gimpl and Fahrenholz, 2001; Ross et al., 2009). Alternatively, others have proposed that the observed somatodendritic release within the hypothalamus is sufficient to activate receptors in distant brain regions by diffusion (Morris and Pow, 1991; Sabatier et al., 2007; Ludwig et al., 2002). These mechanisms are not mutually exclusive and may occur in different brain areas or in response to discrete stimuli.

As mentioned previously, OT regulates lordosis. Central administration of the peptide increases sexual receptivity in hormone-primed and intact females while antagonists and antisense oligodeoxynucleotides to the 5'-region of the human OTR mRNA decrease behavior (Arletti and

Bertolini, 1985; Caldwell et al., 1986; Benelli et al., 1994; Vincent and Etgen, 1993; Witt and Insel, 1991; McCarthy et al., 1994). Mechanisms underlying this behavioral modulation likely involve OTR within the vlVMH, which are upregulated by estradiol (see Figure 1.1) (de Kloet et al., 1986; Insel, 1992; Bale and Dorsa, 1995; Bale et al., 1995). Consequently, the addition of progesterone results in the lateral spread of receptor expression (Schumacher et al., 1989; Schumacher et al., 1990). Additionally, OTR mRNA exhibits a high degree of colocalization with ERα mRNA within VMH neurons, and both the rat OT and OTR genes contain an estrogen response element (ERE) (Devidze et al., 2005; Peter et al., 1990; Adan et al., 1991; Bale and Dorsa, 1997). Finally, OT increases the firing activity of large numbers of vlVMH cells, and neuronal activity is further increased by administration of estradiol (Kow et al., 1991). These findings demonstrate the importance of OT in the lordosis circuit and the ability of the peptide to interact with ovarian hormones in the vlVMH. Chapter Three of this dissertation addresses the subcellular localization of OT and its role in ovarian hormone-induced neural plasticity. Specifically, I explore whether vlVMH dendrites receive differential input according to hormone treatment and OT labeling.

ER action

Historically, it was accepted that mechanisms of estrogen receptor function involve genomic action. ERα is necessary for lordosis behavior and is located in the cell nucleus (Rissman et al., 1997). It is a member of the superfamily of steroid receptors which function as ligand-dependent transcription factors (Beato, 1989). Estradiol is lipophilic and can readily enter cells, where it binds the receptor, the complex dimerizes and then interacts with the DNA sequence within the promoter region of the gene referred to as an estrogen response element (ERE). This is followed by the recruitment of factors that promote transcription and gene expression (Micevych and Christensen, 2012; Cheskis et al., 2007). Evidence of this mechanism in reproductive behavior is supported by results in which lordosis behavior is prevented by drugs that block mRNA or protein synthesis (Rainbow et al., 1982; Yahr and Ulibarri, 1986).

Fairly recently it has become apparent that at least some actions of steroid hormones are nongenomic given their actions that are initiated within seconds or minutes, which seem to be more rapid than can be supported by genomic mechanisms. For example, in the vlVMH, estradiol treatment increases phosphorylation of DARPP-32, which is a signal transduction pathway normally associated with membrane receptors (Auger et al., 2001). Likewise, depolarizing responses of VMH neurons occur quite rapidly (Minami et al., 1990; Kow et al., 2006).

In the context of female reproductive behavior, both estradiol membrane signaling and genomic actions are necessary. The function of the former is to potentiate the latter (Kow and Pfaff, 2004; Vasudevan and Pfaff, 2008). Some membrane actions necessary for lordosis may be mediated by membrane-associated ERα, which can functionally signal through G-protein coupled receptors (GPCRs) (Micevych and Kelly, 2012; Micevych and Mermelstein, 2008; Pedram et al., 2006; Boulware et al., 2007; Boulware and Mermelstein, 2009; Grove-Strawser et al., 2010). Alternatively, the membrane ER Gq-ER has been associated with lordosis behavior (Qiu et al., 2003; Kuo et al., 2010).

As evidence for the significance of membrane ER signaling grows, its specific role in the lordosis circuit and within the vlVMH will be important to determine. The ovarian hormone-induced neural plasticity in the vlVMH (discussed above) has primarily been studied after a four-day regimen of hormone administration known to induce lordosis. Therefore, a picture of the receptive female brain has begun to emerge. However, earlier changes may also be present, including plasticity and signaling that is likely mediated by membrane-associated ERs. Chapter Four tests the hypothesis that proteins associated with spinogenesis in the vlVMH are rapidly regulated by estradiol treatment, which is indicative of membrane ER action.

VMH and lordosis in prairie voles

Most of the work discussed thus far, which establishes that ovarian hormones induce neural plasticity and neuromodulator action in the vlVMH to control female sexual behavior, has been determined via study or laboratory rats. Studying lordosis using rats as an animal model has

many benefits; rats are easy to maintain and manipulate, and have extremely reliable behavioral output with a reasonable similarity and application to other species. Additionally, many aspects of the lordosis circuit and VMH have been well described in this species. However, it is important to demonstrate whether those findings are applicable to other species and whether convergent or divergent behaviors correlate with similarities or differences in neuroanatomy or mechanisms. In order to determine whether dendritic changes driven by ovarian hormones underlie the evolutionarily conserved behavior of lordosis, a species comparison is useful.

Prairie voles are a good candidate species for comparison. They are rodents closely related to rats and exhibit lordosis behavior, but have very different reproductive patterns. Female prairie voles undergo induced estrous, in which exposure to male pheromones causes estradiol secretion. Estradiol then permits copulatory behavior, which in turn stimulates induced ovulation and progesterone secretion. Twenty-four to forty-eight hours of cohabitation with a male also results in a pair-bond after which the male and female exhibit a partner preference for one another, remain socially monogamous, and engage in biparental care of their young (Carter and Getz, 1993). These mating patterns are all in stark contrast to those of rats. Female rats experience spontaneous estrous and ovulation, care for their young without a male, and both male and female rats have multiple sexual partners.

The general circuitry that contributes to pair bonding and mating in female prairie voles is also different from that involved in female rat sexual behavior. Pheromones are more involved in prairie vole mating than in rat reproduction. Therefore, cohabitation with a male activates the vomeronasal organ, which projects to the olfactory bulb, where levels of norepinephrine and gonadotropin releasing hormone change. Secretion of luteinizing hormone from the pituitary into the blood then stimulates the ovaries to produce hormones that allow for mating and impregnation (Carter and Getz, 1993).

The VMH circuitry involved in lordosis, however, may be similar to that in female rats. The role of the VMH in prairie vole behavior is not as well characterized as it is in rats, but it has been indicated to be important. Estrogen receptor alpha (ERα), which is required for female mating

in several other species, is expressed in the prairie vole VMH as well (Fowler et al., 2005; Hnatczuk et al., 1994). Furthermore, it is more densely expressed in females than males (Yamamoto et al., 2006). Cohabitation with a male or exposure to pheromones in his urine activate VMH neurons in female prairie voles (Cushing et al., 2003). Thus, the VMH likely mediates female sexual behavior in prairie voles. However, characterization of the cytoarchitecture of VMH cells in this species is lacking. Specifically, I hypothesize that dendritic architecture of female prairie vole vlVMH neurons is similar to that of female rats. Such conservation would be indicative of functional significance of the specific morphology. Prairie vole dendritic tree and its reliance upon mating status are examined in Chapter Two.

Questions addressed in this thesis

Despite the numerous studies focusing on the role of estradiol and progesterone in the VMH during lordosis, there are a number of details that remain undefined. For example, although many modulators of lordosis have been identified, how they interact with and relate to one another and how they are involved in the observed neuroplasticity is not clear. Importantly, molecular mechanisms underlying ovarian hormone-induced changes in dendritic morphology have not been defined, nor has the functional significance of these structural changes been established, including their reproducibility in other species. These mechanisms and consequences of hormonally regulated dendritic changes are the focus of this thesis. Clarifying some of these unanswered questions will provide insight into the neural effects of ovarian hormones with the goal of enhancing existing knowledge relating to varying central and peripheral systems that utilize differential receptor action and molecular mechanisms to affect diverse and complex health issues. In addition, this work aims to contribute to the understanding of the biologically significant, evolutionarily conserved behavior of lordosis.

The focus of this dissertation is to investigate the behaviorally relevant effects of estradiol on neural plasticity of vlVMH dendrites by testing the following hypotheses: 1) dendritic morphology is correlated to mating status in a species that exhibits lordosis but also rare mating patterns; 2)

vlVMH dendrites that extend into the LFC receive differential input based on hormone treatment and localization of OT; 3) estradiol rapidly increases the phosphorylation of the actin-associated protein cofilin and regulates AMPAR subunit expression in the vlVMH.

CHAPTER 2: Dendritic arbor of neurons in the hypothalamic ventromedial nucleus in female prairie voles (*Microtus ochrogaster*)

Abstract

The ventromedial nucleus of the hypothalamus, particularly the ventrolateral subdivision (vlVMH), has been associated with female reproductive behavior in a variety of species. In particular, it controls lordosis in female rats via ovarian hormone-induced changes in the dendritic tree. Such changes have not been characterized in female prairie voles, rodents that also exhibit lordosis, but also display vastly different mating strategies including induced estrus and ovulation, social monogamy, and bi-parental care. Thus, we examined the dendritic morphology of VMH neurons in this species. Sexually naïve adult female prairie voles were housed with a male for 48 hours to activate the females' reproductive endocrine system. After this cohabitation period, the pair's behavior was observed and level of sexual interest was assessed. Following this interaction, females' brains were processed for Golgi analysis and vlVMH dendritic arbor was examined. Female prairie voles have dendritic trees remarkably similar to those of female rats, but possess significantly longer long primary dendrites. A subset of female voles paired with males did not display sexual activation, and these females had shorter long primary dendrites than the females that exhibited reproductive activation. Lengths of long primary dendrites were positively correlated with plasma estradiol levels in females exposed to males, but not in unpaired females. Further analysis will be necessary to determine the mechanisms responsible for dendrite length and sexual activation; however, this study demonstrates an association between ventrolateral VMH dendrite morphology and female mating behavior in prairie voles, similar to what has been observed in female rats.

Introduction

The ventromedial nucleus of the hypothalamus (VMH) is well documented to control female reproductive behavior in female rats. The hallmark of their sexual receptivity is the reflexive posture lordosis (Griffin and Flanagan-Cato, 2011 and Flanagan-Cato, 2011). Not surprisingly, the VMH has been associated with lordosis in other rodents as well, including mice, guinea pigs, and hamsters. Importantly, however, the VMH also regulates sexual behavior in a wide variety of non-rodent species such as cats and sheep (see Flanagan-Cato, 2011 for review). In fact, electrophysiological and fMRI studies have shown VMH activity during proceptive and receptive stages of non-human primate female sexual behavior (Aou et al., 1988) and when human females are exposed to an androgen-like odorant (Savic et al., 2001). Even rare vertebrates that reproduce asexually exhibit the lordosis posture under the control of the VMH (Grassman and Crews, 1986; Kendrick et al., 1995). Therefore, the VMH is a fundamental component of reproduction and its function has been conserved evolutionarily. Given the evolutionary and biological significance of the VMH, in that it has been well conserved and promotes propagation of genes, characterization of the neuroendocrine and neuroanatomical elements of this nucleus is important.

As the role of the VMH is fairly consistent across species, so are a number of features within the nucleus, such as the expression of estrogen receptors (Flanagan-Cato, 2011). However, dendritic morphology of VMH neurons and hormone-induced neuroplasticity have been mainly described in rats and have not been studied in other species. Specifically, VMH neurons of female rats have relatively simple dendritic trees, with one long primary dendrite, two to three short primary dendrites, and one to two secondary dendrites (Calizo and Flanagan-Cato, 2000 and Griffin and Flanagan-Cato, 2008, Griffin and Flanagan-Cato, 2011). Furthermore, these dendrites in the vlVMH are regulated by ovarian hormones (Frankfurt et al., 1990; Madeira et al., 2001; Griffin and Flanagan-Cato, 2008). Estradiol-induced dendrite length changes in the VMH have been demonstrated in female Syrian hamsters as well (Meisel and Luttrell, 1990). Such cytoarchitectural alterations are likely necessary for the expression of female sexual behavior

across species. Observations in other animal models will be an important step in elucidation of ovarian hormone action in the brain.

Like rats, prairie voles are rodents that display lordosis. However, prairie voles exhibit an unusual mating strategy amongst mammals (Carter and Getz, 1993). Whereas many mammals undergo spontaneous estrus and ovulation, ovarian action in female prairie voles occurs in response to social and chemosensory interaction with non-related males (Carter and Getz, 1993; Carter et al., 1980). Thirty-six to 48 hours of cohabitation with a male result in an increase in circulating estradiol level, uterine weight, and estrogen receptor expression in the brain (Carter et al., 1980 and Cohen-Parsons and Carter, 1987). Mating then occurs and stimulates ovulation (Curtis, 2010). Thereafter the pair will display a life-long social preference for each other (Carter and Getz, 1993; Carter et al., 1980; Witt et al., 1988). Most females become pregnant 2 to 3 days after pairing with a male (Curtis, 2010) and both partners demonstrate bi-parental care of their offspring (Witt et al., 1988).

Data on VMH morphology is scarce in prairie voles, but the nucleus has been implicated in female sexual behavior based on immediate early gene expression in response to exposure to a male (Cushing et al., 2003) and estrogen receptor expression (Hnatczuk et al., 1994; Fowler et al., 2005; Yamamoto et al, 2006). Thus, it is likely that prairie voles would share some hormone-related mechanisms and VMH neuroanatomical features with rats despite their differing reproductive systems. Here, we test this hypothesis by characterizing female prairie vole vlVMH dendritic morphology via Golgi impregnation. Specifically, are the vlVMH dendritic trees of female prairie voles similar to those of female rats and are they associated with mating status as they are in other rodents?

Materials and methods

Animals

Animals were housed in USDA approved facilities with general animal care provided by Laboratory Animal Resources personnel. Sexes were housed in separate rooms at 21°C with a 14:10 light: dark cycle. All animal handling procedures, experimental manipulations, and behavioral testing were approved by the Oklahoma State University Center for Health Sciences Institutional Animal Care and Use Committee.

Subjects were sexually naïve adult female prairie voles from a laboratory breeding colony descended from an Illinois population. Breeders were housed in plastic cages (20 × 25 × 45 cm) containing corncob bedding with hay as nesting material. *Ad libitum* food (rabbit chow supplemented with sunflower seeds) and water were available. Prairie voles appear to be stressed by isolation (Kim and Kirkpatrick, 1996); thus, after weaning at 21 days of age, offspring were housed in same-sex pairs in plastic cages. Subjects were at least 54 days of age when used in experiments.

Behavioral testing

Housing with another female is important to minimize stress in female prairie voles (Grippo et al., 2007); thus, the control group was comprised of females that were housed with unfamiliar females rather than being exposed to males. These females, designated as *unpaired* (UP; $n = 6$), controlled for the novelty associated with exposure to an unfamiliar conspecific. These females were killed at various times (six to 11 min) after removal of the barrier to control for varying time between barrier removal and the time of death of the experimental females.

The experimental group consisted of unrelated male/female pairs of voles that were placed together in standard housing cages and allowed to interact normally. After 24 h, the pairs were separated using a wire mesh barrier (1 cm mesh size) that divided the cage into two equal-sized spaces for a further 24 h prior to behavioral testing. The barrier permitted visual, auditory,

chemical, and limited physical contact, but prevented mating. Behavioral assessment consisted of removing the mesh barrier and assessing the intensity of the males' responses to the females as indicators of the females' reproductive states, as well as the females' behavior toward the males. Pairs were observed carefully during this time to document the behavioral interaction. Pairs were separated at the first indication of lordosis behavior by the female, prior to intromission, to ensure that genital stimulation would not interfere with our primary dependent measures, or after 15 minutes, whichever came first.

Females were grouped into behavioral categories for analysis based on these observations. One group of females was considered to be *reproductively activated* (RA; $n = 5$) because their male partners showed intense interest, including consistently following, continuously anogenitally investigating, and attempting to mount them. Females in this group rarely displayed defensive "boxing" behavior to repel the males' advances. A separate group of females was considered *not reproductively activated* (NA; $n = 4$) because their male partners displayed little sexual interest, instead spending most of their time exploring the cage. Females in this group displayed defensive behaviors that served to deter those male advances that did occur. Thus, males' courtship was influenced by attractivity cues and females' defensiveness or solicitation. The dyadic interaction was unambiguously different between the RA and NA groups. The behaviors of a small number of male-female pairs ($n = 3$) towards one another were intermediate between those of the RA and NA groups, and thus were difficult to accurately categorize. These animals were not included in the final analysis.

At the first sign of imminent copulation, after 15 minutes of interaction with a male, or after a variable time with another female (see above), females were removed from their cages, asphyxiated with CO_2, and decapitated. An initial set of females was used to ensure that the presence of the barrier during cohabitation did not interfere with female endocrine responses to contact with a male. Trunk-blood samples from these females were collected, placed immediately on ice, and then centrifuged at 10,000 rpm for 15 min. The plasma fraction was collected and then stored at -80°C until assayed for circulating estradiol. In addition, uteri from

these females were removed, cleared of surrounding fat, and weighed. To better control for possible differences in the length of the uterine horns, a one-cm section of uterine horn immediately distal to the uterine bifurcation was removed, blotted dry, and weighed to the nearest 0.1 mg. A second set of females was used for analysis of VMH dendrite morphology, in addition to assessments of behavior and estradiol levels. Brains from these females were rapidly excised when the animals were killed, as in the first set of females, above, and placed into 20 ml plastic vials containing fixative for Golgi staining (see below). Trunk blood samples for estradiol assay also were collected from females in this group. When animals were killed, trunk-blood samples were collected, placed immediately on ice, and then centrifuged at 10,000 rpm for 15 min. The plasma fraction was collected and then stored at -80°C until assayed for circulating estradiol.

Estradiol enzyme-linked immunosorbent assay (ELISA)

Each plasma sample was assayed in duplicate with 25 µl used for each replicate. Assays were performed using an Estradiol ELISA kit (Calbiotech #ES180s-100; rabbit polyclonal antibody, detection limit < 3 pg/ml) according to the manufacturer's established procedures. A Biotek Synergy 2 microplate reader with Gen 5 software quantified the absorbance at 450 nm. The averages of the two replicates for each sample (intra-assay variance = 5.01%) were used in statistical analyses. All samples were measured in a single assay.

Golgi impregnation and morphological analysis

Brains were quickly removed and prepared according to the FD Rapid GolgiStain™ Kit User Manual (FD NeuroTechnologies; Ellicot City, MD). Brains were incubated in a potassium dichromate, mercuric chloride, and potassium chromate solution for 2 weeks. After this incubation, VMH sections (100 µm) were obtained using a vibratome (Vibratome® Series 1000). Sections then were placed onto gelatin-coated slides, stained with cresyl violet, dehydrated in ethanol, and coverslipped using Permount® (Fisher Scientific).

Sections containing the VMH were viewed with a BX50 microscope (Olympus; Central Valley, PA). Neurons were considered to be in the ventrolateral VMH (vlVMH) if they were located within the borders of the subdivision according to clear demarcations visible with the Cresyl violet staining (Fig. 2.2A). This study focused on neurons in the vlVMH, based on previous studies that this is the most sexually dimorphic and hormone-sensitive region (Calizo and Flanagan-Cato, 2000 and Dugger et al., 2007). Neurons throughout the rostral-caudal and dorsal-ventral vlVMH were represented in this study (Fig. 2.3). Neurons within the vlVMH then were visualized at 1000X and traced using camera lucida. Next, the tracings were scanned using a Hewlett-Packard Scanjet 3970 and dendrite length was measured using ImageJ. Only neurons in which the entire dendritic arbor could be visualized within a single 100-μm section were analyzed. All morphological analyses were performed with the experimental group concealed.

Dendrites were categorized as either primary or secondary based on whether they extended directly from the soma or branched from a primary dendrite, respectively. Primary dendrites were further categorized based on length, as in previous studies (Calizo and Flanagan-Cato, 2000; Calizo Flanagan-Cato, 2002; Griffin and Flanagan-Cato, 2008). For each neuron, the longest primary dendrite on each neuron was identified as the long primary dendrite (LPD). By definition, each neuron possessed only one LPD. All other primary dendrites were considered to be “short primary dendrites”. Secondary dendrites were those that branched from primary dendrites. After classifying dendrites into these categories, dendrites were determined to be ramifying in one of four directions: dorsomedial, ventromedial, dorsolateral, or ventrolateral (Calizo and Flanagan-Cato, 2000). See Fig. 2.4 for example cameral lucida drawing and dendritic tree characterization.

We analyzed a minimum of eight neurons per animal, each with clearly visible dendrites, for all sixteen animals. Dendrite length for each dendrite type was calculated based on an average of triplicate measurements. The dendrite length for the short primary dendrites was averaged when there was more than one of this dendrite type on a given neuron. The same procedure was followed for secondary dendrites. Values for each animal were calculated as an

average of the values per neuron. The *n* presented in the results reflects the number of animals per group.

Data analysis

Two of the estradiol measures were below the detection limits of the assay and these measures were assigned zeros for statistical analyses. One estradiol measurement from the RA group was beyond three standard deviations from the group mean and was excluded as an outlier. After significance was detected by analysis of variance (ANOVA), group comparisons were probed further using a Newman–Keuls test.

To determine whether dendrite directions were different than predicted by chance, a χ^2 test was employed. A student's t-test was used to test the *a priori* hypothesis that dendrite length would differ between RA versus NA animals. A student's t-test was also employed to compare rat and prairie vole dendrite lengths. Regression analysis was performed to examine the correlations between estradiol levels and the length of LPDs after we verified that all estradiol and LPD values were within 2 standard deviations of the mean. Averages are expressed as the mean ± standard error. Statistically significant differences were inferred when *p*-values were less than 0.05.

Results

The female prairie voles in these experiments weighed 33.1 ± 0.9 g when killed after behavioral testing, and there were no differences in mean body weight between reproductive status groups ($F_{2,25} = 0.64$, $p = 0.53$). In the first set of females, our behavioral categorization was consistent with observed group differences in circulating estradiol levels and uterine weight. Six of the nine females that were paired with males were reproductively activated, whereas one displayed no activation. The responses of these females established that our cohabitation

procedure allowed for sexual activation in female voles, and also defined the relationship between circulating estradiol and uterine weight, an indicator of estradiol action, in these animals. In this first set of females, there was a significant positive correlation between plasma estradiol and uterine weight ($F_{1,12} = 40.44$, $p < 0.0001$, $R^2 = 0.77$; Fig. 2.1A).

As shown in Fig. 2.1B, a similar pattern was seen in animals in set 1 and set 2, in terms of a relation between behavioral category and plasma estradiol levels. In particular, there was a significant effect of estradiol level on reproductive status ($F_{2,24} = 18.42$, $p < 0.001$), and pair-wise comparisons showed that females in the RA group had significantly higher circulating estradiol than did either the NA or UP groups ($p < 0.002$ in both cases). Within each behavioral group, the NA and UP groups did not differ ($p = 0.20$).

Having identified an endocrine correlate of reproductive behavior, we next characterized the basic dendrite morphology of vlVMH neurons in female prairie voles in a second set of animals. The VMH was clearly demarcated in the female prairie voles by cresyl violet stain, as shown in Fig. 2.2A. A total of 130 Golgi-impregnated neurons in the vlVMH were included in the final analysis, with their approximate locations illustrated in Fig. 2.2B. A representative prairie vole vlVMH neuron and its camera lucida representation are shown in Fig. 2.2C. The morphological features of the prairie vole vlVMH neurons were similar to those previously observed in the rat vlVMH (Table 2.1) (Calizo and Flanagan-Cato, 2000 and Calizo and Flanagan-Cato, 2002). On average, there were three to four primary dendrites per neuron, with approximately one branch point in each dendritic tree. In most cases, the LPD extended in the ventrolateral direction. A notable difference was that, despite having smaller brains, prairie voles display much longer dendrites compared with those of rats.

We then evaluated the VMH dendritic tree according to reproductive status. As shown in Fig. 2.3A, reproductive status did not affect dendrite number in the female prairie voles. Likewise, the lengths of the short primary dendrites and secondary dendrites were similar across the three treatment groups (Figs. 2.3B and C, respectively). With regard to the proportion of LPDs that extended in different directions (ventrolateral; ventromedial; dorsolateral; dorsomedial),

regardless of reproductive status, approximately 40-60% of LPDs extended in the ventrolateral direction, and less than 25% extended in each of the other three directions, as shown in Fig. 2.3D.

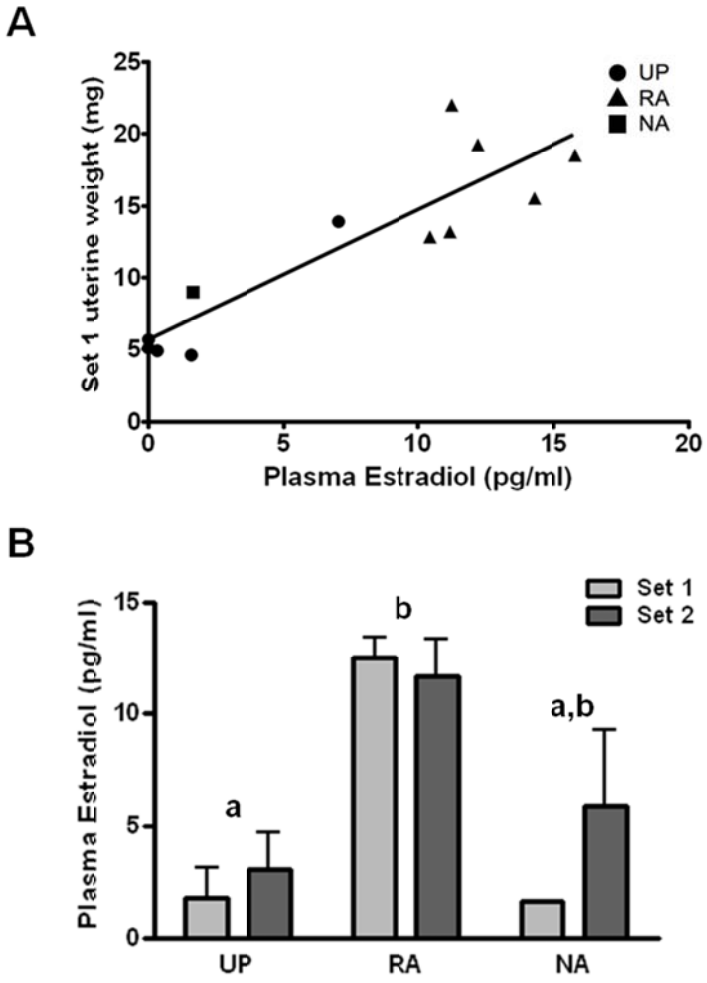

Figure 2.1. Positive relationship between plasma estradiol levels and uterine weights in female prairie voles.
(Panel A). Plasma estradiol concentrations for each of the three reproductive status categories for female prairie voles: unpaired controls (UP); reproductively activated after 48 h of cohabitation with a male (RA); and not reproductively activated after exposure to a male (NA). In panel B, symbols indicate that estradiol levels in the RA group are significantly higher than the UP group, $p<0.001$; the NA group did not differ from either the UP or RA group.

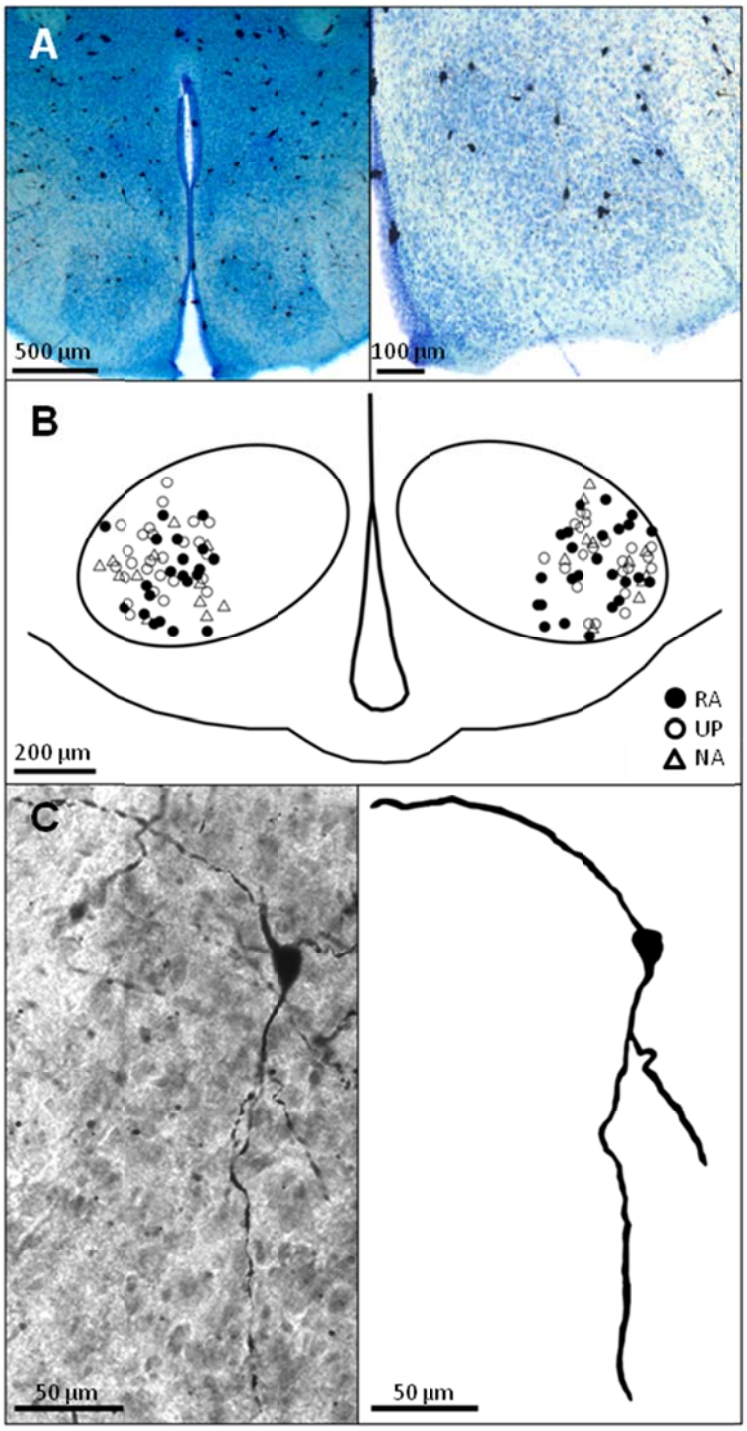

Figure 2.2. Cresyl violet in the VMH, neuron locations, representative neuron drawing. Panel A: low-power photomicrographs of the prairie vole VMH stained with cresyl violet and Golgi labeling. Panel B: a diagram of the prairie vole VMH that summarizes the location of neurons analyzed in the study, coded for the three experimental groups. Panel C: a representative Golgi-labeled VMH neuron, with the associated camera lucida tracing.

	Rat[a]	Prairie Vole
Dendrites per neuron	3.2	3.4
Primary dendrites per neuron	2.6	2.5
Predominant LPD direction	Ventrolateral	Ventrolateral
LPD length (μm)	144	568*
SPD length (μm)	64	245*
Secondary length (μm)	43	113*

Table 2.1. A comparison of the dendritic arbor of VMH neurons in female rat versus prairie vole.
[a] Data for rat are from previous studies and are reproduced here to facilitate species comparisons (Calizo and Flanagan-Cato, 2000, 2002).

In contrast, the length of the LPD co-varied with reproductive status. We compared the RA and NA groups, based on our *a priori* hypothesis that dendrite length would be correlated with reproductive activation. When compared directly, LPDs from reproductively activated females were approximately 20% longer than those from the non-activated females ($p < 0.04$, Student's *t*-test; Fig. 2.4A). Importantly, the length of the LPDs was correlated with plasma estradiol levels in the reproductively activated and non-activated animals (Fig. 2.4B; $F_{1,7} = 8.1$, $p < 0.005$, $R^2 = 0.72$). For the unpaired animals, there was no correlation between LPD length and plasma estradiol level ($p = 0.31$; data not shown). The correlation was not driven by extremes at each end of the range of values given that all values are within two standard deviations of the mean.

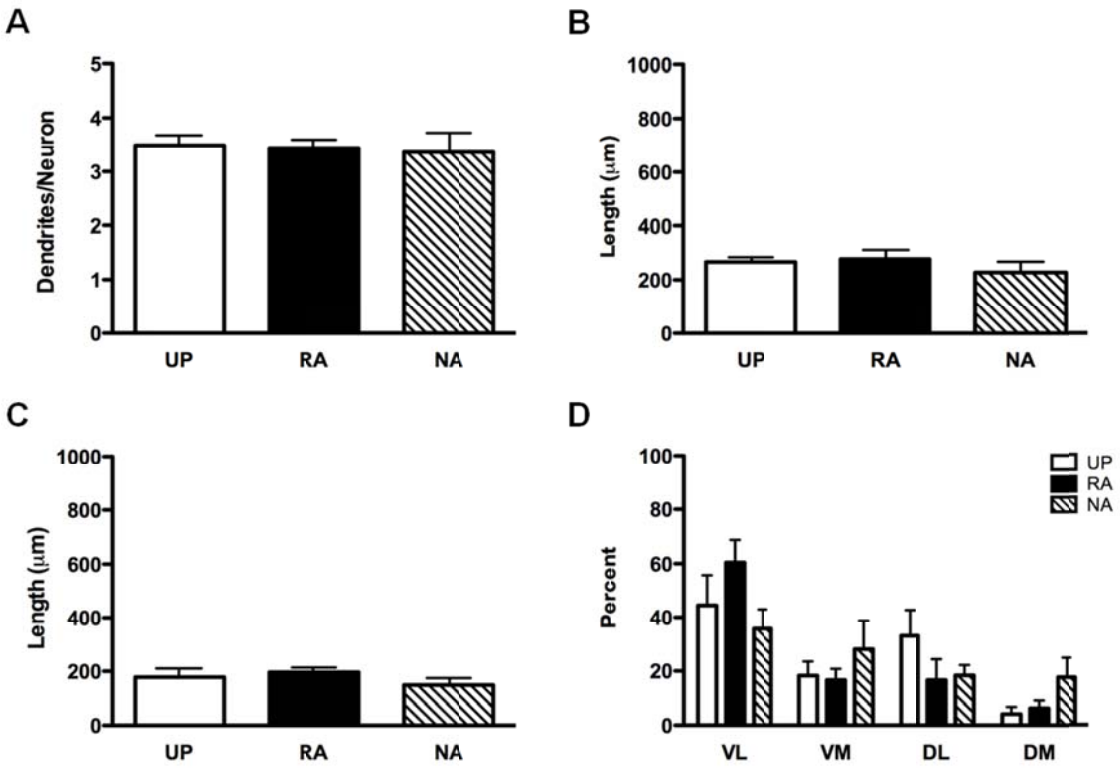

Figure 2.3. Dendrite number and extension direction across groups.
Bar graphs depicting the number of dendrites per neuron in female prairie voles (Panel A); the length of short primary dendrites (Panel B); the length of secondary dendrites (Panel C); and the percent of long primary dendrites extending in the ventrolateral (VL), ventromedial (VM), dorsolateral (DL) and dorsomedial (DM) directions. There was no effect of mating status on any of these parameters in the ventrolateral portion of the hypothalamic ventromedial nucleus. Abbreviations: UP=unpaired; RA=reproductively activated; NA=not activated.

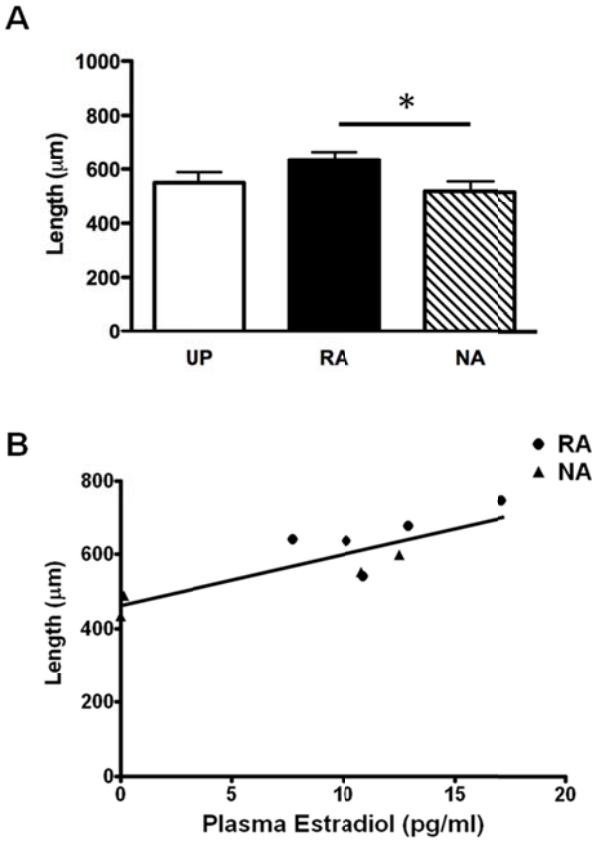

Figure 2.4. Mating status is correlated with dendrite length.
Panel A: bar graph depicting the effect of mating status on the length of long primary dendrites (LPDs) in the ventrolateral portion of the hypothalamic ventromedial nucleus. The LPDs of the reproductively activated (RA) females were significantly longer than those of the non-activated (NA) females ($p<0.04$). The values for the unpaired females (UP) were intermediate, and not different from either of the other two groups. Panel B: the correlation between plasma estradiol level and LPD length in the RA and NA animals ($R^2=0.72$; $p<0.0005$). There was no significant correlation between estradiol levels and LPD length in the UP group (data not shown).

Discussion

The first aim of this study was to compare the dendritic tree of VMH neurons in prairie voles, a rodent with a markedly different mating system, with our previous findings in rats. Wild female Norway rats live in colonies, mate in a polygamous fashion, undergo spontaneous ovarian cycles, and protect and feed their offspring without males' assistance. In contrast, female prairie voles live monogamously, demonstrate mate-induced ovarian activation, and engage in bi-parental care of their offspring. Despite these pronounced differences in mating systems, most parameters of the dendritic arbors of VMH neurons were quite similar in these two rodents. A notable and striking exception was the length of the dendrites, which were two to four times longer in prairie voles than has previously been reported for rats.

The second goal of this investigation was to test the hypothesis that, in prairie voles, the lengths of LPDs on vlVMH neurons vary according to reproductive status, as has been observed in female rats. Indeed, we observed that the reproductively activated group, defined largely by the high level of interest of the male partners during the behavioral test, exhibited elevated estradiol levels and longer LPDs compared with the females that were not reproductively activated. Intriguingly, the non-activated group, initially identified by the lack of attention from their male partners during the behavioral test, exhibited less evidence of ovarian activation (i.e., changes in plasma estradiol levels) as a group and shorter LPDs in the VMH, two measures that were highly correlated. These key findings will be discussed in turn.

VMH dendritic arbor in rat versus prairie vole

Comparative studies offer unique opportunities to understand mechanisms that are either concordant or divergent with a behavior that is either shared or varies across species. Although the overall regulation of mating behavior is substantially different between rats and prairie voles, females of the two species display mating postures that are virtually identical. Given that certain cytoarchitectural elements within VMH, namely the number and types of dendrites arising from

VMH neurons, are conserved across these two species, we propose that these elements may govern the lordosis posture.

Because of the prominent similarity between the dendritic trees in these species, it will be important to extend similar comparative studies to other species. The VMH has been implicated in female mating behavior in a broad range of vertebrates, including lizards, birds, rodents, ruminants, carnivores, and primates (Aou et al., 1988, Blache et al., 1991, Goy and Phoenix, 1963, Kendrick et al., 1995, Leedy and Hart, 1985, Malsbury et al., 1977, Rand and Crews, 1994,Sterner et al., 1992 and Takahashi and Lisk, 1985). Nevertheless, the dendrite morphology of the VMH neurons has been described in only a few species, most extensively in rats (Calizo and Flanagan-Cato, 2000, Frankfurt et al., 1990, Griffin and Flanagan-Cato, 2008, Madeira et al., 2001 and Millhouse, 1979–1981), and to a lesser extent in hamsters (Meisel and Luttrell, 1990). In female prairie voles, the VMH expresses estrogen receptors and VMH neurons are activated by social contact (Cushing et al., 2003 and Hnatczuk et al., 1994). Taken together, these data suggest that, as in rats, the prairie vole VMH integrates hormonal and social cues that are important for the expression of mating behavior. This interpretation is consistent with the observations by Hnatczuk et al. (1994) of somewhat additive effects of estrogen replacement and exposure to males in inducing sexual behavior in ovariectomized female voles. Future studies may test this possibility by extending the cross-species comparison of the cytoarchitecture of neurons in the lordosis circuit, comparing organisms with dominant environmental versus endogenous controls of reproductive behavior.

The singular dissimilarity uncovered in this study was a marked difference in dendrite length; female prairie voles have significantly longer dendrites than rats despite the fact that voles have smaller brains. This may reflect a trend for prairie vole neurons to require increased extranuclear input in order to process the more complex social cues involved in their mating system. Alternatively, the lateral fiber complex (LFC), which in rats lies adjacent to the VMH and likely provides afferent input and neurochemical substrates to the nucleus that contribute to the regulation of lordosis, may differ qualitatively in prairie voles. At present, this fiber plexus has not

been described in prairie vole literature; a more thorough investigation is needed to help clarify this possibility.

Another possibility for the variation in dendrite length in prairie voles versus rats could be attributed to differences in lifetime exposure to estradiol, rather than to a species difference. The rats used in previous studies had undergone puberty and multiple spontaneous estrous cycles. In contrast, the voles were hormonally naïve until our experiment began. It would be important for future studies to examine whether the initial exposure to estradiol differentially affects dendrites compared with subsequent exposures.

Regulation of dendrite length in prairie voles

The important similarity between females of both species revealed in the present study is the dependence of the long primary dendrite length on mating status. Here, the reproductive status of female prairie voles was manipulated by housing sexually naïve females with males for 48 h. Although this experimental procedure resulted in reproductive activation of most females, a few females were categorically dissimilar, and thus were classified as being non-activated. This is to be expected, as approximately 25% of female prairie voles typically do not respond immediately to exposure to a male (Curtis, 2010 and Witt et al., 1988). These within-species differences provide a unique opportunity to examine natural variation in the processes that underlie reproductive activation. Previous studies reported a positive relationship between plasma estradiol levels and sexual activation in female prairie voles (Cohen-Parsons and Carter, 1987). Our finding of low levels of circulating estradiol and corresponding low uterine weights in the non-activated group is consistent with those studies, and verified that our behavioral assessment reflected a physiological difference. Interestingly, the subset of females for which exposure to males failed to produce ovarian activation (i.e., no increase in plasma estradiol or uterine weights) displayed shorter LPDs in the VMH than did those from activated females.

Given the correlational nature of this study, determining the direction of causality will require further investigation. Nonetheless, several scenarios are possible. In rats and hamsters,

ovarian hormone levels are indicative of reproductive status and regulate dendrite length in the VMH (Griffin and Flanagan-Cato, 2008, Madeira et al., 2001 and Meisel and Luttrell, 1990). Thus, one possibility is that LPD length in voles is regulated by changes in gonadal hormone levels as has been shown in other species (Griffin and Flanagan-Cato, 2008 and Meisel and Luttrell, 1990). Our finding that dendrite length and circulating estradiol were positively correlated would, on the surface, appear to support such a possibility. However, Griffin and Flanagan-Cato (2008) reported that elevated estradiol decreased, rather than increased, LPD length in rats. This suggests that, if estrogen regulates VMH dendrite length in sexually naive voles, the effects are opposite to those seen in rats. It should be noted that the Griffin study also reported that progesterone treatments subsequently restored the dendrites of estrogen treated females to lengths equivalent to those of control females. This latter effect is unlikely to have contributed to the present results because progesterone is not necessary for sexual behavior in voles (Cohen-Parsons and Carter, 1987) and, in fact, does not increase until after mating has occurred (Carter et al., 1987). It must be emphasized that the female voles in the present study were experiencing their first exposure to changes in gonadal hormones. In contrast, the rats in previous studies likely had experienced several estrous cycles prior to testing (Griffin and Flanagan-Cato, 2008). Thus it is possible that the "rat-like" pattern of gonadal hormone regulation of LPD length may arise in the voles after they too have experienced multiple fluctuations in gonadal hormones. In this light, it would be informative to examine LPD length in female voles during the species-typical post-partum estrous. Further, a lack of estradiol effects on LPD length does not preclude other types of dendritic remodeling such as changes in dendritic spine density (Calizo and Flanagan-Cato, 2000).

A second scenario is that, rather than responding to changes in circulating estradiol, the LPDs contribute to the control of estradiol secretion. This idea is supported by the observations of axonal projections from the VMH to GnRH neurons (Goubillon et al., 2002), providing an anatomical substrate for this proposed effect. Thus, the individual differences in the lengths of the LPDs that we found in the voles may explain the variability in sexual activation observed in

this and previous studies (e.g., (Curtis, 2010)). The length of the LPDs may affect the sensitivity to cues from the male that promote estradiol release, and thereby, sexual receptivity. As such, even those females that expressed shorter LPDs would eventually become sexually activated, given enough time with her partner. In fact, previous research has suggested a wide range of latencies for the onset of sexual behavior (Curtis, 2010 and Witt et al., 1988), leading us to consider this the most likely explanation. The LPDs may process information from the accessory olfactory system, given that a functional vomeronasal organ and accessory olfactory system are critical for initiating sexual behavior in voles (Curtis et al., 2001; Lepri and Wysocki, 1987; Solomon et al., 1996). Information from the olfactory pathways ultimately reaches the VMH via projections from the vomeronasal amygdala and the bed nucleus of the stria terminalis. It would be of great interest to examine whether the length of the LPDs in female prairie voles is correlated with their ability to display the Bruce effect (Stehn and Richmond, 1975), another olfactory-mediated process (Bellringer et al., 1980).

Caveats

At present, we have limited information about the behavioral differences between the reproductively activated and non-activated females. For example, there may have been subtle group differences in behavior between the initial social pairing and the behavioral testing that may require extremely fine-grained analysis to detect. Further study also may reveal that the non-activated females would become fully activated with prolonged exposure to a male. This seems likely, as plasma estradiol levels were correlated with dendrite length in the RA and NA females, but not in the UP females, likely a result of the heterogeneous nature of the group; presumably, once paired with a male, a subset of the UP females would become activated and some percentage would not, as has been demonstrated in previous studies. Thus, we propose that dendrite lengths of naïve females are variable but regulated by contact with a male, and exposure time necessary for elongation and subsequent reproductive activation depends upon individual differences in female neuroanatomy or male pheromones. We also cannot rule out the possibility

that females failed to become sexually activated due to some deficit in the males or incompatibility with specific males. Such a possibility has been raised by the observation that about half of non-responsive female prairie voles become sexually active when paired with a second male (Curtis, 2010). The present results suggest, however, that deficits in the males likely play a minimal role in the delayed female activation. A more likely explanation is that the reproductively delayed females were those with shorter LPDs in the VMH, and the re-pairing of females with a second male simply provided a sufficiently long male exposure for activation to occur.

To avoid an effect of mating itself on neuronal morphology in the VMH (Flanagan-Cato et al., 2006), we placed a wire mesh barrier between the male and female after the initial 24 h of cohabitation (i.e., during the time when the natural onset of sexual receptivity would be expected to occur). The behavioral and hormonal measures in most females suggest that the barrier did not inhibit reproductive activation. It may be useful in future studies to allow mating to proceed, providing indisputable evidence of behavioral activation and a more discrete dependent measure for correlational analysis with neuronal morphology.

The present study used Golgi impregnation to analyze the dendritic arbor. Limitations of this method include the capricious nature of Golgi labeling, the lack of neurochemical markers, and the possible loss of dendrite elements due to the plane and thickness of the brain sections. We were able to circumvent the lack of a prairie vole brain atlas by counterstaining the brain sections with Cresyl violet, which provided a vivid demarcation of the VMH. It is unknown whether the combination of Golgi impregnation and this counterstain would work as well in other species. Given the labor-intensive nature of camera lucida drawing, the number of animals in some groups is somewhat low; however, this shortcoming is offset somewhat by the inclusion of eight neurons per animal.

In a broader perspective, the current study focused on the role of the VMH in female mating behavior; however, this nucleus has been implicated in male sexual behavior as well, including inter-male aggression (Lin et al., 2011). It would be interesting to examine sex and

species differences in VMH dendrite morphology in the context of these other behaviors under its control.

Significance

The results indicate that co-variation between VMH dendrite morphology and sexual receptivity, previously observed in laboratory rats, is also observed in prairie voles, a species with a markedly different regulation of mating behavior. The data from the non-activated females suggest that dendrite length is closely linked with reproductive activation, rather than with social exposure *per se*. Additional investigation is needed to demonstrate any causal links between elevated estradiol, VMH dendrite structure, and behavior in female prairie voles. As has been noted previously (Curtis, 2010), this study identified a subset of females that failed to become reproductively activated after social exposure to a male. At present, the underlying cause for the failure in reproductive activation is not known; however, we have identified several possible reasons that can be tested in future studies.

The phenomenon of non-activation in female voles may provide an interesting model system for the study of the biological basis of hypoactive sexual desire disorder. The role of the LPDs in the VMH in reproductive activation is an exciting avenue for future research. The fact that dendrites within the VMH may represent a neurological marker for individual differences in sexual response can provide a logical starting point.

Conclusions

Here I have established a conserved effect of importance of ovarian hormone-sensitive dendrite length in mating behavior in female prairie voles, which employ a very different mating system from female rats. In the next chapter I return to the rat model system to investigate synaptic organization in the lateral fiber complex (LFC), the area into which vlVMH dendrites extend in these two species. Utilizing immunoelectron microscopy, I analyzed ultrastructure of

the LFC and explored the effects of ovarian hormones on the synaptic organization in that area. Finally, I characterized the localization and hormone modulation of the neuropeptide oxytocin (OT), which is important for reproductive behavior.

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Author contributions

LMF, JTC, and SLF designed research; JTC, CJR, KSC, and SLF performed research; JTC, KSC, SEW and SLF analyzed data; LMF, JTC, and SLF wrote the paper.

CHAPTER 3: Ovarian hormone-induced reorganization of oxytocin-labeled dendrites and synapses lateral to the hypothalamic ventromedial nucleus in female rats

Abstract

The neuropeptide oxytocin (OT) has been implicated in a variety of social behaviors in a number of brain regions. One such biologically important social behavior is female sexual receptivity, marked by the display of lordosis. This copulatory posture is regulated by ovarian hormones and modulated by OT action in the ventrolateral subdivision of the ventromedial nucleus of the hypothalamus (vlVMH). Our group has shown that vlVMH dendrites extend into the lateral fiber complex (LFC) that lies adjacent and provides afferent input, including OT, to the vlVMH. In addition, we have demonstrated for the first time that vlVMH dendrites make synaptic contact with axonal boutons from the LFC. The length of these vlVMH dendrites is regulated by ovarian hormones. Thus, the subcellular localization of OT and synaptic reorganization that accompanies dendrite retraction from the LFC may be important for the expression of female sexual behavior, but has not yet been described. Using immunoelectron microscopy, I test the hypotheses that 1) dendritic profiles in the LFC that originate in the vlVMH receive differential synaptic input based on hormonal status as well as presence or absence of OT, and 2) that the presence of oxytocin in a vlVMH dendrite extending into the LFC is related to the type of input (oxytocinergic/glutamatergic versus non-oxytocinergic) it receives.

Introduction

The interaction between a male and female rat during the mating process may be a brief social interaction, but it is an important one nonetheless. Female sexual behavior has long been studied because of its utility as a model for central ovarian hormone action. An integral part of female sexual receptivity, the hallmark of which is lordosis, is ovarian hormone-induced neuroplasticity in the ventrolateral subdivision of the ventromedial nucleus of the hypothalamus (vlVMH) (see Flanagan-Cato, 2011 for review). Specifically, estradiol has been shown to increase axodendritic synapses and spine density, and regulate dendrite length (Carrer and Aoki, 1982; Nishizuka and Pfaff, 1989; Frankfurt and McEwen, 1991; Frankfurt et al., 1990; Madeira et al., 2001; Calizo and Flanagan-Cato, 2000; Griffin and Flanagan-Cato, 2008).

In particular, estradiol decreases the length of long primary dendrites extending from the vlVMH into the adjacent fiber plexus (lateral fiber complex; LFC) (Griffin and Flanagan-Cato, 2008; Griffin et al., 2010). This fiber field contains few cell bodies. Instead, it is comprised of an intricate array of axonal fibers that contain a number of transmitters and neuromodulators that regulate lordosis behavior (Moss and McCann, 1975; Kow and Pfaff, 1988; Vincent and Etgen, 1993; Zemlan et al., 1973). Because of the paucity of afferents that penetrate the vlVMH, it is likely that the extension and retraction of the long primary dendrites from the vlVMH over the estrous cycle are crucial for forming synapses with axons in the LFC. The resulting synaptic connections would allow the nucleus to receive input from the LFC that is necessary for behavior (Millhouse, 1973; Griffin and Flanagan-Cato, 2008; Griffin et al., 2010; Flanagan-Cato, 2011). Thus, this complex reorganization is extremely important for ovarian hormone action in the vlVMH and female sexual behavior. However, little is known mechanistically about the events that precede and follow it.

The estradiol-induced decrease in vlVMH dendrite length, which is reversed within four hours of progesterone administration, likely leads to subsequent reorganization of synapses in the LFC. Likewise, modifications in afferent input and changes in modulators of lordosis are a

plausible result. The localization or amount of molecules present in the LFC that may be affected after synaptic reorganization, which contribute to the lordosis circuit, include norepinephrine, serotonin, gonadotropin releasing hormone, and oxytocin (OT) (Millhouse, 1973; Merchenthaler et al., 1984; Schumacher et al., 1989; Swanson and Hartman, 1975; O'Donohue et al., 1979; Joseph et al., 1981; Palkovits, 1982; Watts et al., 1987; Daniels and Flanagan-Cato, 2000; Flanagan-Cato, 2011 for review). In the present study, we focus on the involvement of oxytocin in ovarian hormone-induced synaptic reorganization in the LFC.

Oxytocin is a highly conserved nonapeptide, variations of which are present in virtually all vertebrates (du Vigneaud et al., 1953; Gimpl and Fahrenholz, 2001 for review). It has been associated with a variety of social behaviors, including maternal behavior, pair-bonding/romantic attachment, peer association, social learning, memory, and anxiety. It has been linked to human trust, immune function, pain perception, and ingestive behavior as well (see Gimpl and Fahrenholz, 2001; Lee et al., 2009; Ross and Young, 2009 for review). In addition, numerous studies have shown that oxytocin is necessary for lordosis behavior. OT administered intracerebroventricularly (icv) significantly increased lordosis in ovariectomized females pretreated with physiological or subthreshold doses of estradiol and progesterone (Arletti and Bertolini, 1985; Vincent and Etgen, 1993) or only estradiol in other studies (Caldwell et al., 1986), as well as in intact females in estrus (Benelli et al., 1994). Correspondingly, icv administration of an oxytocin antagonist or parenchymal infusion of antisense oligodeoxynucleotides to the 5'-region of the human oxytocin receptor (OTR) mRNA into the VMH significantly blocked behavioral effects of oxytocin in steroid-primed ovariectomized (OVX) females; both receptive and proceptive behaviors were decreased and rejection behaviors were increased (Witt and Insel, 1991; Pedersen and Boccia, 2002; McCarthy et al., 1994). Also, sexual activity significantly increased the transcription factor FOS, a marker of neuronal activity, in the VMH in the vicinity of the OT fibers in the LFC (Flanagan et al., 1993).

Further support for its behavioral importance, OT action is regulated by ovarian hormones. Oxytocin receptors are upregulated by estradiol in the vlVMH (de Kloet et al., 1986).

The subsequent addition of progesterone causes an expansion of the area of OTR expression in the LFC (Schumacher et al., 1989; Schumacher et al., 1990). Autoradiography revealed that cycling animals showed similar increases in OT binding when hormone levels were high (Insel, 1992; Johnson et al., 1989). *In situ* hybridization data complemented these findings by demonstrating hormone-induced increases in OTR mRNA in exogenously treated as well as intact females (Bale and Dorsa, 1995; Bale et al., 1995). Finally, OT has been shown to increase firing activity in large numbers of vlVMH cells, and neuronal activity was further increased by administration of estradiol (Kow et al., 1991). Therefore, OT is integral to the lordosis circuit within the vlVMH and LFC.

Although it is clear that OT has an important role in female sexual receptivity, the mechanisms by which it exerts its effects are not clear. As mentioned previously, OTR modulated by ovarian hormones are present in the vlVMH and LFC, but the source of OT had not been previously defined, as no OT-producing neurons have been found within the VMH. The peptide is produced primarily in the hypothalamic supraoptic and paraventricular nuclei (SON and PVN, respectively) (Schumacher et al., 1989). These distant nuclei send projections to the posterior pituitary via fibers within the LFC. Our lab first documented synaptic connections in this area, providing evidence that some of the LFC fibers have axons that provide oxytocinergic input to vlVMH dendrites that extend into the LFC.

In this first ultrastructural description of the LFC, myelinated axons were identified by the presence of microtubules, neurofilaments, and the characteristic myelin lamellae. Oxytocin was located within these myelinated axons, but not in unmyelinated axons. An analysis of the area uncovered that myelinated axons were less numerous than dendritic profiles and axonal boutons. As expected, cell bodies were sparse in the region (Griffin et al., 2010). In addition, OT labeling was present in axonal boutons, and 100% of OT-containing boutons made synaptic contact. These observations led us to investigate the types of synapses present, and the frequency in which they occurred, discussed later in this chapter.

Unexpectedly, OT was also observed in dendrites, recognized by the occurrence of microtubules, ribosomes, and mitochondria, and confirmed by double-labeling for the dendrite-specific marker MAP2. The presence of OT in dendrites ramifying from the vlVMH was surprising because, as previously mentioned, no OT is produced in the VMH. An estradiol-induced reduction in dendrite density in the LFC was observed, which was reversed by the addition of progesterone (Griffin et al., 2010). Importantly, this finding utilized a different methodology and replicated the results from an earlier Golgi impregnation study. Griffin and Flanagan-Cato (2008) had previously revealed an estradiol-induced decrease in the length of long primary dendrites that extend out from the vlVMH into the LFC, an effect reversed by progesterone. Thus, the complex and significant change in vlVMH dendrite arrangement caused by ovarian hormones was reproduced. However, the current method utilizing electron microscopy allowed us to further investigate the presence of OT particles and configuration of axonal boutons, which provided additional information about synaptic organization in the LFC and how it is affected by ovarian hormones.

Additional analysis revealed that the density of boutons was unaffected by hormone treatment. However, OT-immunoreactive boutons tended to make asymmetric synaptic contacts with dendrites, suggestive of excitatory neurotransmission. Interestingly, all OT-containing boutons were also labeled with vesicular glutamate transporter VGLUT2, indicating glutamatergic action (Griffin et al., 2010). This finding may serve as a significant first step in understanding the dynamics of OT and glutamate transmission. Colocalization in presynaptic boutons and co-release of these two elements have been observed in other areas of the brain (Peters et al., 2008; Hrabovszky et al., 2006; Ponzio et al., 2006). However, co-release in the vlVMH/LFC would be surprising, as glutamate attenuates, while OT facilitates lordosis behavior (McCarthy et al., 1991; Georgescu and Pfaus, 2006a; Arletti and Bertolini, 1985; Caldwell et al., 1986; Benelli et al., 1994; Vincent and Etgen, 1993).

This ultrastructural characterization of the LFC provided a great deal of important and novel information about the area, but questions of the effects of the ovarian hormone-induced

reorganization on pre-and post-synaptic LFC elements and oxytocin localization remained. Therefore, in my follow-up analysis, I examined the

and tested the hypothesis that estradiol and progesterone modulate the synaptic organization in the LFC, particularly with regard to OT-labeled synaptic elements. Specifically, I expected that vlVMH dendrites that did not retract from the LFC in response to estradiol would receive increased synaptic input and that OT would be a marker of this reorganization.

Materials and methods

Animals

A total of nine adult Sprague-Dawley female rats (Taconic, Hudson, NY) were group housed in plastic tubs (41 x 21 x 22 cm) with standard bedding. Rat chow and tap water were available ad libitum. The colony temperature was maintained at 22ºC, with a 12/12-hour reverse light/dark cycle, and with lights off at 1100 hours. After they had acclimated to the colony for one week, animals were bilaterally ovariectomized during general anesthesia (90 mg/kg ketamine and 9 mg/kg xylazine, both intraperitoneally, (i.p.). Upon completion of the surgery, rats were given yohimbine (2.1 mg/kg, i.p.) to counteract the xylazine and, after a period of observation, were returned to their home cages. The Institutional Animal Care and Use Committee of the University of Pennsylvania approved all animal procedures.

After one week of recovery from the ovariectomy, animals were randomly assigned to one of three hormone treatment groups: vehicle (veh), estradiol benzoate (EB) alone, or EB combined with progesterone (EBP). Animals were administered two daily subcutaneous injections of sesame oil (vehicle) or 10 µg of 17-β-estradiol benzoate (Sigma, St. Louis, MO; both EB and EBP groups). Forty-eight hours later, the vehicle and EB groups were administered vehicle (propylene glycol) and the EBP group received 500 µg of progesterone (Sigma). All animals were deeply anesthetized four hours after the last injection and transcardially perfused

through the ascending aorta with a series of solutions: 10 ml heparinized saline, 50 ml of 3.3% acrolein (Electron Microscopy Sciences, Fort Washington, PA), and 200 ml of 2% formaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed immediately after the perfusion fixation, and postfixed in the final solution overnight at 4 ºC. Sections then were cut in the coronal plane (40 µm) by using a Vibratome (Technical Product International, St. Louis, MO) and collected into 0.1 M phosphate buffer.

Immunoelectron microscopy

The immunoelectron microscopy study used a polyclonal antibody generated in rabbit (T-4084; Bachem, King of Prussia, PA; 1:1,000 dilution) raised against the nonapeptide OT (H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2). Radioimmunoassay data provided by the manufacturer reported 100% and 0% cross-reactivity for OT and vasopressin, respectively. Coronal sections throughout the rostrocaudal extent of the VMH were processed for electron microscopic analysis after immunolabeling for OT only, or double labeling for OT with VGLUT2 or MAP2. In double-labeled sections, immunoperoxidase labeling was used to identify MAP2 or VGLUT2, whereas silver-intensified immunogold labeling was used to identify OT. Briefly, sections were placed for 30 minutes in 1% sodium borohydride in 0.1 M phosphate buffer (pH 7.4) to reduce amine-aldehyde compounds. For all incubations and washes, sections were continuously agitated with a rotary shaker. The tissue sections then were incubated in 0.5% bovine serum albumin in 0.1 M Tris-buffered saline (TBS; pH 7.6) for 30 minutes. After this incubation, thorough rinses in 0.1 M TBS were performed. Subsequently, sections were incubated in rabbit anti-OT (Bachem; 1:1,000 dilution) alone or in a cocktail containing mouse anti-VGLUT2 (1:500 dilution) or mouse anti-MAP2 (1:500 dilution) in 0.1% bovine serum albumin. Sections were incubated for 15–18 hours on a rotary shaker at room temperature. The following day tissue sections were rinsed three times in 0.1 M TBS and incubated in biotinylated donkey anti-rabbit (1:400; Vector, Burlingame, CA) and biotinylated donkey anti-mouse (1:400; Vector) for 30 minutes followed by rinses in 0.1 M TBS. Next, sections were placed in an avidin-biotin complex (Vector) for 30 minutes. This

antibody-enzyme complex was visualized by a 10-minute reaction in 3,30-diaminobenzidine (0.22%; Sigma-Aldrich) and hydrogen peroxide (0.3%) in 0.1 M TBS. After primary anti-OT incubations, sections were rinsed three times with 0.1 M TBS, followed by rinses with 0.1 M phosphate buffer and 0.01 M PBS, pH 7.4. Sections then were incubated in a 0.2% gelatin-PBS and 0.8% bovine serum albumin buffer for 10 minutes followed by incubation in goat anti-rabbit immunoglobulin conjugated with 1-nm gold particles (Amersham Bioscience, Piscataway, NJ) at room temperature for 2 hours. Sections then were rinsed in buffer containing the same concentration of gelatin and bovine serum albumin, as above. After rinses with 0.01 M PBS, sections then were incubated in 2% glutaraldehyde (Electron Microscopy Sciences) in 0.01 M PBS for 10 minutes. Next, sections were washed in 0.01 M PBS and 0.2 M sodium citrate buffer (pH 7.4). A silver enhancement kit (Amersham Bioscience) was used to intensify the immunogold labeling. The optimal times for silver enhancement ranged between 10 and 15 minutes. Some tissue sections incubated for the detection of OT and synaptophysin or VGLUT2 were reverse-labeled such that OT was labeled with immunoperoxidase, and synaptophysin or VGLUT2 were labeled with silver-intensified immunogold to confirm a lack of cross-reactivity. After intensification, tissue sections were rinsed in 0.2 M citrate buffer and 0.1 M phosphate buffer and incubated in 2% osmium tetroxide (Electron Microscopy Sciences) in 0.1 M phosphate buffer for one hour, washed in 0.1 M phosphate buffer, dehydrated in an ascending series of ethanol followed by propylene oxide, and flat-embedded in Epon 812 (Electron Microscopy Sciences) on aclar (Electron Microscopy Sciences). Sections from all three groups were processed in parallel.

Coronal sections of the LFC were cut with a diamond knife (Diatome-US, Fort Washington, PA) by using a Leica Ultracut ultratome at a thickness setting of 74 nm to obtain ultrathin sections. Images of the selected sections were compared with light microscopic images of the block face before sections were taken. Ultrathin sections were collected on copper mesh grids, examined with a Morgagni 268(D) transmission electron microscope (FEI, Hillsboro, OR), and digital images were captured by using the Advanced Microscopy Techniques (Danvers, MA)

Advantage HR charge-coupled device (CCD) camera system. Figures were assembled and adjusted for brightness and contrast in Adobe Photoshop (San Jose, CA).

Data analysis

For image analysis, digital images were obtained from the rostral and midrostral portions of the LFC from all brains, all with good preservation of ultrastructural morphology. For each animal, three distinct 40-μm coronal sections were assayed. From each of these sections, four copper grids, spaced three grids apart and each containing three to four ultrathin sections, were analyzed. For the occasional case in which the appropriate grid was damaged, the next available grid was analyzed. Thus, 12 copper grids were analyzed per animal.

Images were captured with a range of objectives (22,000-44,000x). Figures were assembled and adjusted for brightness and contrast in Adobe Photoshop. Experimenters, blind to the hormonal status of individual animals, conducted the subcellular quantification. The inter-rater reliability was 0.91.

The cellular elements were identified based on the description of Peters and colleagues (1991). For example, somata were identified by a nucleus, Golgi apparatus, and smooth endoplasmic reticulum. Dendrites contained endoplasmic reticulum, were postsynaptic to axon terminals, and were larger than 0.7 μm in diameter. A terminal was considered to form a synapse if it showed a junctional complex or a restricted zone of parallel membranes with slight enlargement of the intercellular space, and/or was associated with postsynaptic thickening.

Statistical analysis

For each animal, the quantification of ultrastructural features reflected the average values from three 40-μm sections, each of which included ultrathin sections from four separate copper mesh grids. For all parameters measured, a two-way ANOVA tested for a main effect and an interaction among hormone treatment, subcellular compartment, or the presence of OT labeling. If warranted, a Bonferroni-corrected t-test was employed for post hoc comparisons. Data are

expressed as the mean (±SEM). All statistical comparisons were performed by using the Prism 4.0 statistical software (GraphPad, San Diego, CA).

Results

The trapezoid in Figure 3.1 depicts the area of the LFC analyzed. This region of interest was positioned directly adjacent to the vlVMH and contained OT-immunoreactive fibers. In total, 4,879 dendritic profiles and 1,077 axonal boutons in 702 photomicrographs were characterized. An area of 713.2 (±80.3) mm^2 was analyzed per animal, and the area examined per animal was equivalent across the three treatment groups. Because of the nature of the analysis, only a small portion of the dendrite can be visualized in each photomicrograph. Thus we can only make observations regarding that portions, which will be referred to as a "dendritic profile" throughout this document.

Because dendritic profile density was affected by hormone treatment, but synaptic density was not (Griffin et al., 2010), next synaptic profiles for each dendrite were examined and presence or absence of OT was analyzed. As shown in Figure 3.3A, there was a main effect of the presence of OT in the dendritic profile ($F_{(1,12)} = 39.890$, $p < 0.0001$) and a main effect of hormonal condition ($F_{(2,12)} = 4.653$, $p < 0.040$). Specifically, although the density of OT-positive dendritic profiles did not change across treatment groups (Fig. 3.2), dendritic profiles with OT in the estradiol condition received significantly more synapses than OT-containing dendritic profiles of vehicle treated animals (0.898 ± 0.095 versus 0.590 ± 0.144, $p < 0.050$). On the other hand, ovarian hormone treatment had no effect on number of synaptic contacts per non-OT-containing dendritic profiles. Therefore, those non-OT dendritic profiles that remained after the estradiol-induced decrease underwent no change in average synaptic input (Fig. 3.3A).

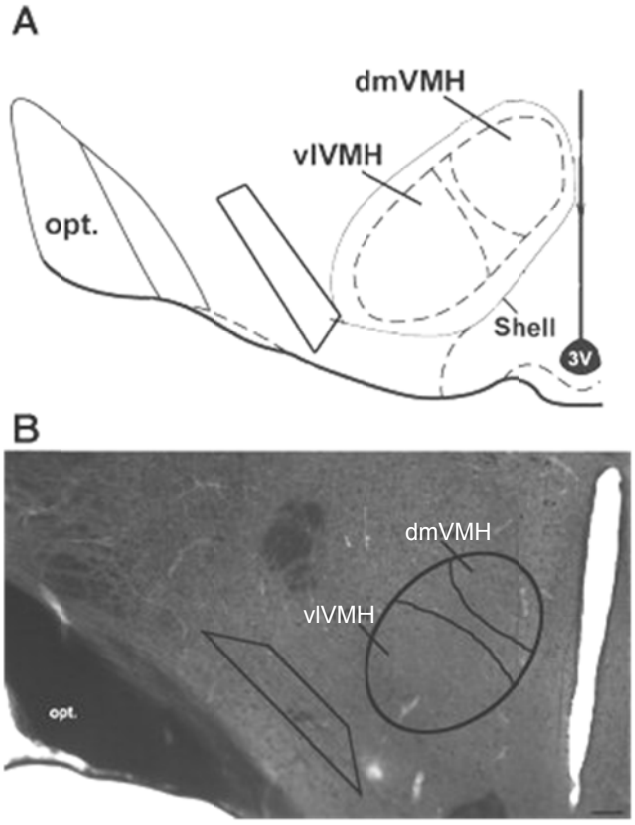

Figure 3.1. Diagram and photomicrograph illustrating the region that was analyzed at the ultrastructural level.
A. An atlas drawing of the mediobasal hypothalamus, including the VMH. The trapezoid indicates the area that was analyzed with the electron microscope. B. A corresponding 40-µm section, immunolabeled with peroxidase for OT and treated with osmium tetroxide for ultrastructural analysis. A trapezoid has been superimposed on the OT fibers, indicative of the area of study. The approximate borders of the dorsomedial ventromedial nucleus of the hypothalamus (dmVMH) and ventrolateral VMH (vlVMH) are delineated. opt, optic tract; 3V, third ventricle. Scale bar = 100 µm in B. Originally published in the dissertation of Gerald D. Griffin, "Ovarian hormones remodel the lattice of dendrites and oxytocinergic innervation of the female rat hypothalamic ventromedial nucleus."

In order to more thoroughly characterize the numbers of synaptic inputs in OT+ versus OT- dendritic profiles, and whether hormone treatment affected this distribution, we quantified the percentage of dendritic profiles with zero, one, two, and three synaptic inputs. Figure 3.3B illustrates the distribution of the number of synaptic contacts per dendritic profile. Two-way ANOVAs were performed for each group with a similar number of synaptic inputs (0, 1, 2, and 3). Many dendritic profiles did not appear to have synaptic input, but of those with no input, a significantly higher proportion of non-OT-labeled dendritic profiles had zero synaptic contacts compared with the OT-labeled dendritic profiles (approximately 73% versus 54% overall) ($F_{(1,12)}$ = 36.93; $p < 0.0001$). There was also a significant main effect of hormone treatment ($F_{(2,12)}$ = 5.120; $p < 0.030$). Conversely, a significantly lower proportion of non-OT labeled dendritic profiles had one (20% versus 27%), two (4% versus 12%), or three (0.5% versus 5%) synaptic contacts compared with the OT-labeled dendritic profiles ($F_{(1,12)}$ = 5.018, 38.53, and 39.93, respectively ($p < 0.050$, 0.0001, and 0.0001, respectively). A Bonferroni post hoc test revealed that estradiol or estradiol plus progesterone treatment further increased the percent of OT-labeled dendritic profiles that received three synaptic contacts compared with vehicle treatment (7.533 ± 1.244 versus 1.500 ± 0.321, $p < 0.001$, and 6.433 ± 1.770 versus 1.500 ± 0.321, $p < 0.010$, respectively, Fig. 3.3B).

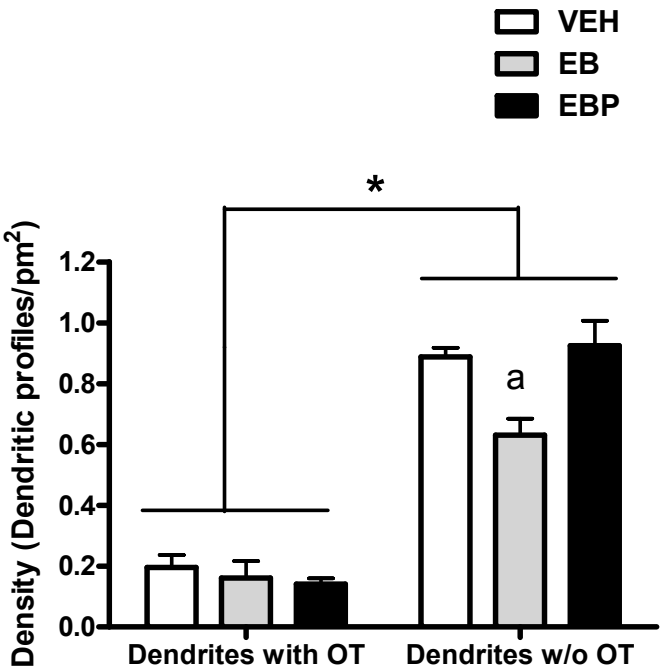

Figure 3.2. Bar graph summarizing the density of dendritic profiles according to whether they were negative or positive for OT immunolabeling.
There was an interaction between ovarian hormone treatment and labeling for OT on the density of dendritic profiles. There was a main effect of OT labeling, with unlabeled outnumbering OT labeled dendritic profiles by approximately 7:1. Although ovarian hormone treatment had no effect on the density of OT-labeled dendritic profiles, estradiol treatment reduced the density of unlabeled dendritic profiles. The effect of estradiol was reversed by subsequent progesterone administration. Brackets indicate differences in density between dendrites with and without OT. Level of significance between OT labeling status is indicated by * ($p < 0.05$) and the effect of estradiol treatment on dendritic profile density, compared with vehicle and estradiol plus progesterone treatments, is indicated by "a" ($p < 0.01$). Abbreviations: VEH, vehicle treatment; EB, estradiol benzoate treatment; EBP, estradiol benzoate and progesterone treatment. Originally published in the dissertation of Gerald D. Griffin, "Ovarian hormones remodel the lattice of dendrites and oxytocinergic innervation of the female rat hypothalamic ventromedial nucleus."

Once it was determined that OT-containing dendritic profiles received more synaptic input, we assessed whether OT+ or OT- dendritic profiles were more likely to make synaptic contact with OT+ or OT- axonal boutons, and whether ovarian hormones affected innervation patterns. In surveying the possible dyads of synaptic partners, we found all four types present: an OT+ bouton innervating an OT+ dendritic profile, an OT+ bouton innervating an OT- dendritic profile, an OT- bouton innervating an OT- dendritic profile, and an OT- bouton innervating an OT+ dendritic profile (Fig. 3.4). As there were no differences across hormone treatment groups between types of dendritic profiles relative to input type (data not shown), the veh, EB, and EBP groups were combined. A two-way ANOVA revealed a significant main effect of input type, with most dendritic profiles receiving no synaptic input ($F_{(2,24)} = 271.600$; $p < 0.0001$). There was also a significant interaction between input type and dendritic profile type ($F_{(2,24)} = 76.860$; $p < 0.0001$). Further analysis revealed that dendritic profiles with no synaptic partners were less likely to contain OT (74.189 ± 1.720 versus 52.879 ± 3.229, $p < 0.001$). Dendritic profiles that were innervated by axonal boutons without OT particles were more likely to exhibit OT labeling themselves (37.889 ± 2.333 versus 23.200 ± 1.652, $p < 0.001$). Finally, OT-labeled synaptic terminals preferentially innervated OT-labeled dendritic profiles versus non-OT-labeled dendritic profiles (9.233 ± 1.915 versus 2.611 ± 0.445, $p < 0.05$, Fig. 3.5). A summary of the main findings is shown in Figure 3.6.

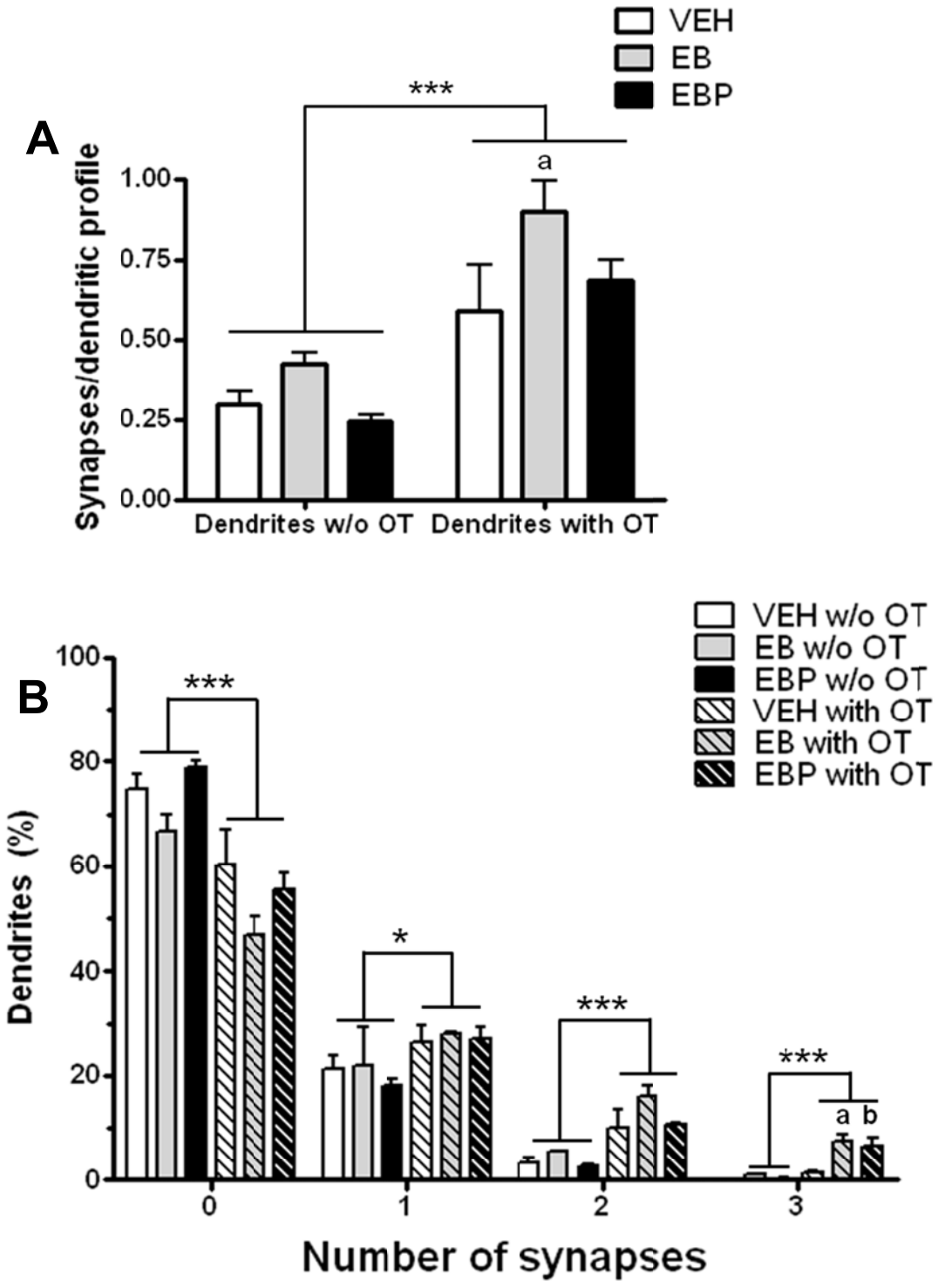

Figure 3.3. Bar graph and histogram summarizing the interaction between the presence of OT in dendritic profiles, ovarian hormone treatment, and the number of synapses per dendritic profile.

A. There was a main effect on the average number of synapses per dendritic profile, with OT-labeled dendritic profiles receiving approximately twice as many inputs as non-OT labeled dendritic profiles. Although ovarian hormones did not alter the level of input to the non-OT labeled dendritic profiles, estradiol treatment increased the number of synaptic contacts per OT-labeled dendritic profile by approximately 52%. *** indicates p <0.0001. a indicates p <0.05 compared to vehicle. B. The histogram illustrates that dendritic profiles with or without OT most commonly were observed to receive no synaptic terminals. OT-labeled dendritic profiles were less likely to have zero synaptic inputs, and more likely to have one, two, and three synaptic inputs, compared with unlabeled dendritic profiles. Estradiol and estradiol plus progesterone treatment increased the percentage of OT- containing dendritic profiles that received three synaptic contacts. Brackets indicate a main effect of OT-labeled versus non-OT labeled dendritic profiles. Level of significance is indicated by * (p < 0.05) and *** (p < 0.0001). Significant effects of ovarian hormones treatments compared with vehicle are indicated by “a” (p < 0.001, compared to vehicle) and “b” (p < 0.01, compared to vehicle). Abbreviations: VEH, vehicle treatment; EB, estradiol benzoate treatment; EBP, estradiol benzoate and progesterone treatment.

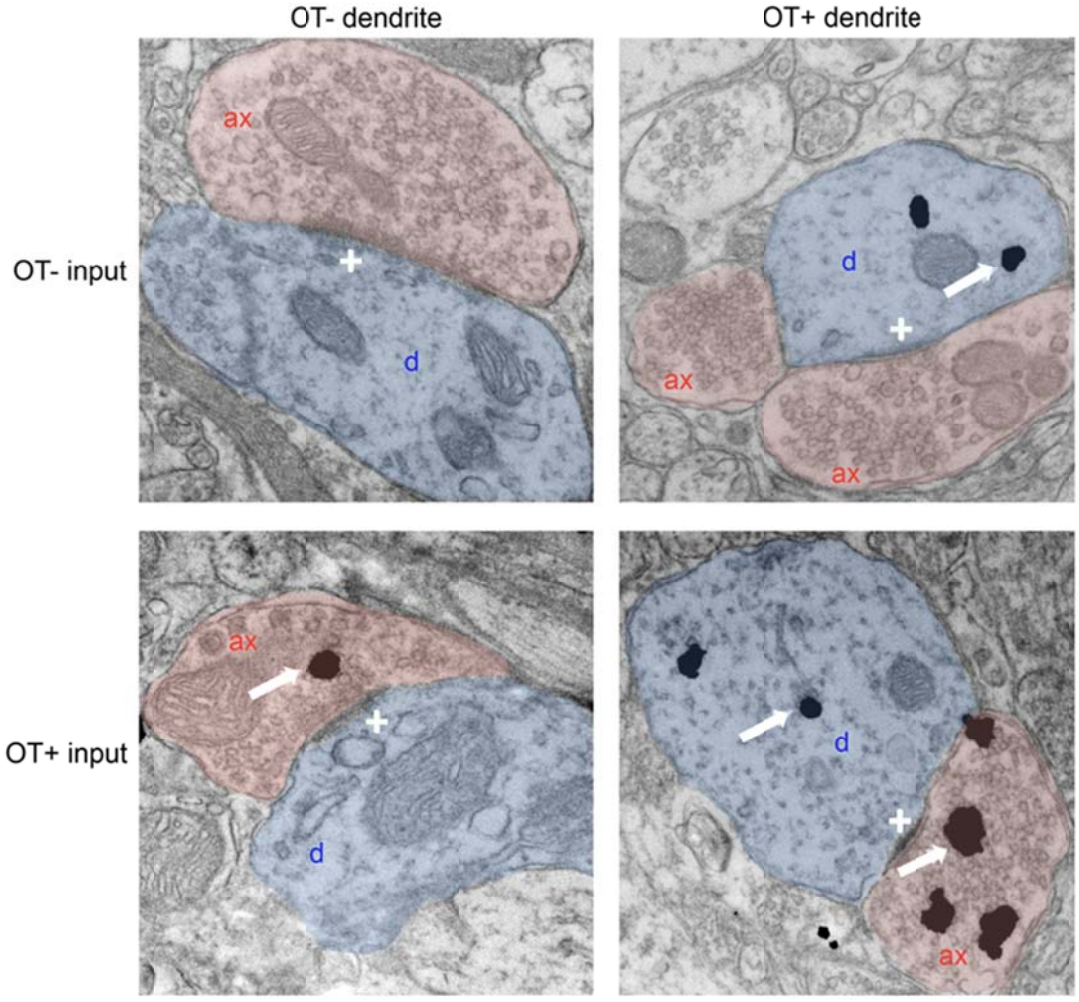

Figure 3.4. Electron photomicrographs illustrating OT-labeled dendritic profiles and axon terminals in the VMH lateral fiber plexus.
Immunoelectron micrographs were prepared and analyzed, as described in Griffin et al. (2010). For most axodendritic synaptic contacts, both the dendritic profiles and the axon terminals were absent of labeling for OT. For some axodendritic contacts, either the dendritic profiles or the axon terminals were labeled for OT, but not both. In a few cases, axodendritic synaptic contacts were observed in which both the dendritic profiles and the axon terminals were labeled for OT. Abbreviations: ax = axon, pseudocolored pink; d = dendritic profile, pseudocolored blue; plus sign indicates an active zone of synaptic contact; white arrows indicate immunogold particles, indicative of OT.

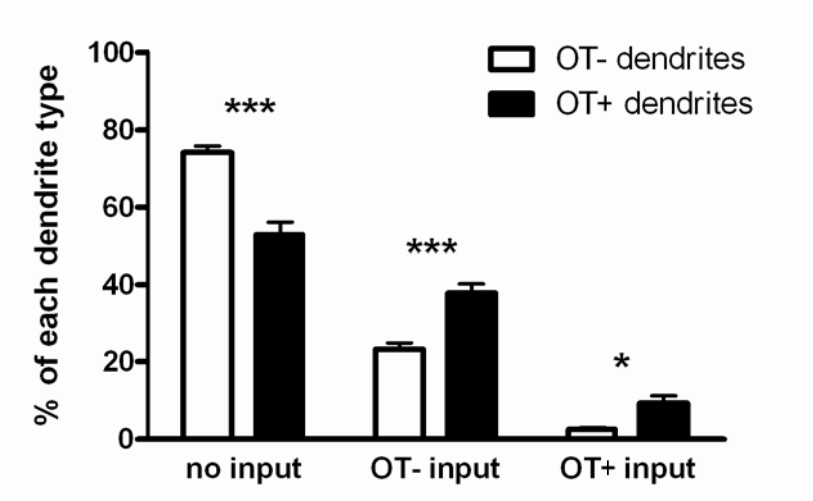

Figure 3.5. Bar graph comparing the pattern of synaptic input to dendritic profiles in the LFC depending on whether the dendritic profile was labeled for OT.
There was no effect of hormone treatment on percentage of dendritic profiles innervated by axonal bouton type, therefore ovariectomized animals treated with vehicle, estradiol alone, and estradiol plus progesterone were combined (n = 9 animals, including a total of 5118 dendritic profiles). OT-labeled dendritic profiles were more likely to have synaptic inputs, including more likely to have OT-labeled synaptic inputs, compared with non-OT-labeled dendritic profiles. * indicates $p < 0.05$; *** indicates $p < 0.001$.

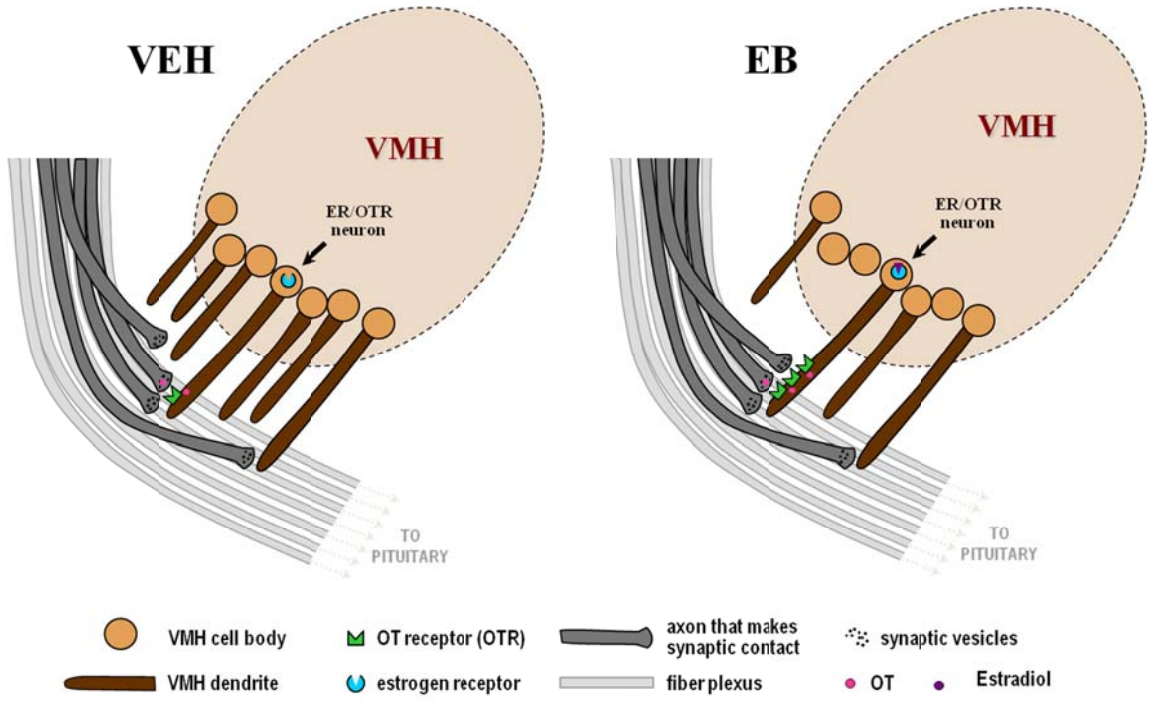

Figure 3.6. Model of estradiol-induced structural organization in the VMH.

In the vehicle condition (VEH), the ratio of dendrites without OT to those with is ~7:1 and dendrites with OT have twice as many synapses as those without. In the estradiol-treated group (EB, right), the number of OTR increases and the density of dendrites without OT decreases ~29% while the density of OT-containing dendrites remains unchanged. In addition, estradiol further increases the synaptic input of OT-containing dendrites.

Discussion

These data, as part of the first ultrastructural study of the fiber complex adjacent to the VMH (LFC), tested the hypotheses that estradiol-induced retraction of dendrites extending into the LFC would result in increased synaptic input in remaining dendrites, the neuromodulator oxytocin is a marker of such neuroplasticity, and progesterone would further alter dendritic input. Here, we reveal OT labeling within dendritic profiles and confirm an estradiol-induced retraction of VMH dendrites. In particular, the effect is specific to non-OT-containing dendritic profiles and is reversed within four hours by progesterone. In contrast, OT-containing dendritic profile numbers remain constant regardless of hormone treatment. In addition, OT-labeled dendritic profiles receive more synaptic input than non-OT-containing profiles, and estradiol treatment causes a further increase in synaptic input specifically in the OT+ dendritic profiles which is also reversed upon addition of progesterone. Figure 3.6 depicts a summary of the changes we observed in the vehicle and estradiol treatment conditions. Finally, because axonal boutons that contain OT are more likely to make synaptic contact with dendritic profiles that also contain OT versus those that don't, and because of OT's position distant from the synapse, it is likely that OT is released parasynaptically and internalized into dendrites.

Effects of ovarian hormones on dendritic profiles

The estradiol-induced reduction of dendritic profile density was an important finding, as it reproduced Griffin and Flanagan-Cato's (2008) results obtained using Golgi impregnation. This previous study showed that the lengths of long primary dendrites extending from the vlVMH were decreased in response to estradiol. The current experiment, utilizing a different method, electron

microscopy, was able to reinforce those findings by showing a decrease in dendritic profile density in the LFC, the area in which the dendrites from vlVMH neurons extended and subsequently retracted. Notably, in both cases, the addition of progesterone reversed the effect of estradiol alone, restoring dendrite length or density to vehicle levels (Griffin and Flanagan-Cato, 2008; Griffin et al., 2010).

It may seem counterproductive for estradiol and progesterone to exert opposite effects on hypothalamic morphology. However, such an effect has been observed before in the medial preoptic nucleus, where estradiol induces mu-opioid receptor internalization, and progesterone reverses the effect (Mills et al., 2004). Such examples likely function as a temporal control such that the female only uses energy to mate when levels of both hormones are high and she has a maximal likelihood of becoming impregnated. Within the vlVMH/LFC area, the likely function of dendrite length changes in response to ovarian hormones is to regulate synaptic contact between vlVMH dendrites and fibers in the LFC, which may provide oxytocinergic and other types of input that promote lordosis behavior. Likewise, parallels between vehicle and estradiol plus progesterone conditions in the Golgi and electron microscopy studies appear contradictory, as they represent opposite ends of the sexual receptivity spectrum. However, it is clear that dendrite length is important for the exhibition of lordosis, but not the only factor. Numerous other ovarian hormone-regulated changes are likely necessary as well. For example, expression of dendritic spines and various receptors and release of neurotransmitters and peptides are all crucial for the display of lordosis.

The role of OT in synaptic reorganization

In addition to reproducing the estradiol-induced dendrite retraction, the present method utilizing immunoelectron microscopy allowed us to differentiate dendrite chemical phenotype, which is not possible with Golgi and other techniques. By labeling for OT, we determined that the dendritic retraction from the LFC is specific to those dendrites that do not contain OT. As OT-containing dendrites are not included in the estradiol-induced dendritic withdrawal from the LFC,

OT appears to be a marker for dendritic plasticity during synaptic rewiring in response to ovarian hormones. It will be an interesting topic of future research to further characterize the OT-containing dendrites. Determining whether they express OT, estrogen, and other receptors may help elucidate a mechanism by which these dendritic profiles are spared estradiol-mediated retraction and what role they might play in female sexual behavior.

Synaptic Input Patterns

Because we observed such dynamic changes in dendritic profile density related to ovarian hormones and OT, but no changes in axonal bouton or synapse density regardless of hormone or OT status, we tested the hypothesis that synapses proliferate on OT-labeled dendrites by examining average synapses per dendritic profile. Dendritic profiles labeled with OT have strikingly higher average numbers of synapses per dendritic profile than those that were not labeled with OT. Furthermore, the increased synaptic input of OT+ dendrites is amplified by estradiol treatment (Fig. 3.3A). Again, because total synapse number remains constant, with the presence of estradiol, boutons likely form new synapses with OT+ dendrites after the retraction of their previous non-OT dendrite synaptic partners (see figure 3.6 for a representation). Likewise, when we examined the breakdown of percentage of dendrites with different numbers of synaptic contacts, dendrites without OT labeling are more likely to receive no input, while dendrites with OT labeling are more likely to have multiple synaptic partners. Finally, both ovarian hormones increase the percentage of dendritic profiles that had three synapses (Fig. 3.3B). Therefore, OT is a marker for specific dendrite types that receive more innervations.

Glutamate in OT-labeled axonal boutons

While these data are indicative of specific estradiol-induced synaptic reorganization wherein OT- dendrites retract and their synaptic partners subsequently make contact with remaining OT+ dendrites, the mechanism underlying this change is not clear, nor are the consequences. Because we know that OT in boutons colocalizes with glutamate (Griffin et al.,

2010), and that these OT+ boutons are more likely to innervate dendritic profiles that contain OT and which remain in the estradiol condition, it is likely that at least some dendrites receive increased glutamatergic input after estradiol treatment. As glutamate in the vlVMH is reportedly inhibitory to lordosis behavior (McCarthy et al., 1994; Georgescu and Pfaus, 2006a), this may be another mechanism by which receptive behavior is attenuated prior to the progesterone action needed for maximal likelihood of successful mating. A proposed role for glutamate transmission involves the fine control of defensive behaviors during mating. In this way, glutamate can regulate pacing in order to increase likelihood of impregnation (Georgescu et al., 2012). At least one previous study has indicated that estradiol increases, and estradiol plus progesterone decreases glutamate in the VMH, which complements behavioral data (Luine et al., 1997). Colocalization of ER and VGLUT2 mRNA in the VMH also suggests that estradiol could affect glutamate transmission (Eyigor et al., 2004). Glutamate levels have not been measured in the LFC, however. We did not measure whether the estradiol-induced increase in synaptic input was specifically glutamatergic, but this should be a focus for future studies, as well as further investigation of glutamate release dynamics under different hormone conditions. Examination of other types of input changes in response to ovarian hormones will also be important.

Possible mechanisms of synaptic reorganization

Alternatively, glutamate may not be strictly inhibitory to lordosis as previously interpreted. Because glutamate and its multiple receptor types are ubiquitous throughout the lordosis circuit, effects of agonists and antagonists may be excessive and not representative of physiological conditions, and thus may be misleading. Spatial and temporal specificity of glutamate action are likely very important to control precise components of female sexual behavior, an idea supported by modest behavioral differences resulting from targeting specific glutamate receptors (Georgescu et al., 2006a; Georgescu et al., 2006b). Thus, details of glutamate signaling during lordosis and possible co-release of OT and glutamate are likely complex and require further investigation.

An additional question remains concerning OT peptide levels. Because of the non-stereological area-sampling methodology of the present study, we were not able to quantify relative amounts of OT across hormone treatment groups, but it would be interesting to know whether there might be more or less OT present in the LFC after estradiol-induced dendrite retraction or whether ovarian hormones caused a change in OT release dynamics. In addition to these proposed experiments, studies aiming to elucidate receptor dynamics will also be crucial. In fact, expression of oxytocinergic fibers is generally well conserved across rodent species, including in rats, mice, and prairie voles. However, the pair-bonding, sexual, and maternal behaviors of these species are quite different, as are their levels of OTR expression. Therefore, differences in receptor expression rather than variation in peptidergic fiber density, correlate with and likely contribute to behavioral discrepancies (Insel, 1992; Olazabal et al., 2006; Ross et al., 2009).

Overall, many dendritic profiles within the LFC have no synaptic input, but this may be due to the small portion of dendrites visualized in each electron photomicrograph. When synapses are observed, all four combinations of dendrite-bouton dyads are present in the LFC: OT- bouton innervating an OT- dendrite, OT+ bouton innervating an OT+ dendrite, an OT- bouton innervating an OT+ bouton, and an OT+ bouton innervating an OT- dendrite (Fig. 3.4). Particularly, though, OT-containing boutons were more than three times as likely to innervate OT+ dendritic profiles as OT- dendritic profiles. Ovarian hormones did not alter these patterns. Thus, while oftentimes an OT+ bouton is in contact with an OT+ dendrite, it is not uncommon for an OT+ dendrite to be proximal to but not innervated by an OT+ bouton (Fig. 3.5). This finding indicates possible parasynaptic release. Moreover, when OT particles were present in boutons, small, clear synaptic vesicles were also present. However, the OT was located away from the synaptic region and apposing dendrite, while the vesicles within terminals were aligned with the junctional zone (Griffin et al., 2010). This is further evidence that OT, like other peptides, is released parasynaptically. Additionally, the surprising discovery of OT in dendrites indicates that

OT may be internalized with its receptor and later recycled. A proposed model is depicted in Figure 3.7.

Alternatives to the internalization hypothesis to explain the presence of OT in dendrites do not seem applicable in the present scenario. As discussed earlier, neither OT mRNA nor OT peptide have been localized to the VMH (Sawchenko and Swanson, 1985; Sofroniew, 1985), and although there are many brain areas that express OTRs, OT is synthesized in relatively few places (Sabatier et al., 2007; Leng et al., 2008). The production primarily takes place in magnocellular hypothalamic neurons of the paraventricular (PVN) and supraoptic (SON) nuclei, and to a lesser extent in parvocellular neurons of the PVN. Thus, the presence of OT in dendrites is not a product of OT-producing neurons within the vlVMH. Likewise, while traditional neuropeptides, including OT, can be dendritically released and OT-producing neurons in the SON and PVN have dendrites long enough to possibly extend into the VMH (Stern and Armstrong, 1998; Griffin et al., 2010), the dendrites analyzed in the present study more than likely originate in the vlVMH and not the PVN or SON. The lack of VGLUT2 labeling, which has been documented in the PVN and SON, in dendrites here precludes the possibility (Ponzio et al., 2006).

Although there is little data about oxytocin receptor (OTR) desensitization and internalization in neurons, work with cell lines expressing OTRs has indicated that they, like many other G-protein coupled receptors, become desensitized after ligand binding and extended stimulation. The receptor may then be endocytosed and internalized, likely via a clathrin-dependent pathway, and targeted to other intracellular compartments for recycling or degradation (Guzzi et al., 2002; Phaneuf et al., 1998; Rimoldi et al., 2003; Conti et al., 2009; Gimpl and Fahrenholz, 2001; Gimpl et al., 2008; Smith et al., 2006). One group has described that human OTR expressed in HEK293T cells are internalized, recycled, and resensitized within four hours via a short cycle involving the small GTPases Rab4 and Rab5 (Conti et al., 2009). However, the mechanism may vary according to brain region. Therefore, further investigation of OTR dynamics and the probable participation of endosomes in the vlVMH *in vivo* are necessary.

As mentioned previously, estradiol plus progesterone administration results in a re-lengthening of vlVMH long primary dendrites extending into the LFC (Griffin and Flanagan-Cato, 2008). Additionally, estradiol plus progesterone results in an extension of oxytocin receptor expression laterally from the VMH (Schumacher et al., 1989; Schumacher et al., 1990). A lesion study confirmed that the oxytocin receptors extending into what we now refer to as the LFC originate from the vlVMH (Johnson et al., 1991). Thus, we may assume that the dendrites that are elongated in this hormone condition contain OTR, providing a mechanism for the observed expansion of expression. However, this seemingly contradicts our hypothesis that OT is present in dendrites because the dendrites express oxytocin receptors and have internalized the peptide. The current data show that OT+ dendrites (which we assume to express oxytocin receptors) do not retract after estradiol administration, which in turn implicates that the dendrites that exhibit estradiol-induced reduction in length do not express oxytocin receptors. Therefore receptor expression expansion indicates that OTR-expressing dendrites exhibit hormone-mediated length changes, while the presence of OT in dendrites that do not retract indicates that OTR-expressing dendrites do not exhibit hormone-mediated length changes. While these assumptions are interesting to consider, there are other factors most likely involved. It is possible that because of parasynaptic release being somewhat nonspecific, some dendrites devoid of OT labeling may also express oxytocin receptors but did not have internalized OT at the time of observation. In addition, investigating estrogen receptor expression may help provide clues about possible mechanisms involved in this synaptic reorganization, although previous research indicates that colocalization of ERα and OTR is extremely likely; single cell RNA expression in the vlVMH revealed that 86.2% of OT receptor-expressing vlVMH cells also express ERα in intact females, while estradiol-treated OVX females showed 81.8% coexpression (Devidze et al., 2005). This finding serves as evidence that estradiol and OT can both act upon the same cell. Clearly we cannot conclude at this time how many populations of dendrites are present in the vlVMH, including whether or not they express OTRs, and how they each respond to ovarian hormone

treatment, but these findings indicate that the dynamics of hormone-induced dendritic plasticity in this brain area are quite complex and OT is a important part of the mechanism.

Regardless of whether or not OT is internalized into dendrites via OTR, the source of origin is not definitive. Besides the main sources of OT in the PVN and SON, small amounts of OT are also produced in the bed nucleus of the stria terminalis, medial preoptic area, and lateral amygdala then released into the brain (see Gimpl and Fahrenholz, 2001 for review). Additionally, there are many OT-immunoreactive fibers throughout the brain as well, including in the septum, amygdala, hippocampus, subiculum, entorhinal complex, olfactory bulb, dorsal motor nucleus of the vagus, the nucleus accumbens, and the LFC (see Gimpl and Fahrenholz, 2001 for review). Finally, OTR are expressed throughout the brain (Elands et al., 1988; Freund-Mercier et al., 1987; Tribollet et al., 1988; Yoshimura et al., 1993). Consequently, the OT system is complex and in some instances, high levels of OTRs are present without nearby OT-containing fibers (De Vries and Buijs, 1983). Therefore, the source of OT related to some behaviors and brain areas remains unclear. This uncertainty includes the mechanism by which the VMH receives OT to control lordosis.

Several non-mutually exclusive proposed routes of OT action have been proposed. Neuropeptides, including OT, can be released in bulk from dendrites to activate receptors in distant brain regions by diffusion (Ludwig et al., 2002; Pow and Morris, 1989). Such a mechanism would account for cases where there is a lack of alignment between OT source and receptors. Additionally, it has been suggested that neuropeptide-containing dense core vesicles contain high enough concentrations with long half-lives that are able to reach far-away targets effectively (Pow and Morris, 1989; Landgraf and Neumann, 2004; Ludwig and Leng, 2006). However, traveling far from the site of release makes peptide dilution, uptake, and breakdown more likely, and the specific dynamics of OT bulk release targeting the vlVMH have not been described. Importantly, it seems unlikely that a general diffusion method can provide enough specificity of action to act only on the desired receptors and render only particular effects.

Alternatively, the classical hypothesis maintains that magnocellular OT neurons project to the posterior pituitary and parvocellular neurons to various regions within the brain. Recent studies in the nucleus accumbens support this idea. Ross et al. (2009) utilized electron microscopy and retrograde tracing to demonstrate synaptic contact between OT-containing fibers and nucleus accumbens neurons as well as a direct pathway from the PVN and SON to the accumbens, although it is not clear whether these fibers are direct projections or collaterals of axons.

Another possibility involves a combination of the previous mechanisms, with some point-to-point synaptic transmission, and some bulk release diffusion. Combining these possibilities would allow for temporal and spatial specificity in response to a variety of stimuli (Kombian et al., 2002; Landgraf and Neumann, 2004). Determining under which circumstances one method is active versus another may depend on the brain area, the hormonal or chemical status of the animal, or the desired behavior.

While we cannot disregard bulk release, we show synaptic contact in the LFC and both pre- and postsynaptic OT for the first time, suggesting that there is at least some specific transmission involved in OT's modulation of female sexual behavior. Further exploration of LFC fibers will distinguish whether these connections are direct or involve terminals that branch off of pituitary-projecting axons. Finally, while not a focus of the present experiments, there is evidence that OT release from the PVN specifically is involved in lordosis, as sexual activity in estradiol- and progesterone-treated female rats increased FOS immunoreactivity only in the PVN. FOS-IR was also present in the VMH and the OT-immunoreactive LFC (Flanagan et al., 1993).

Conclusions

In conclusion, our proposed mechanism by which OT promotes lordosis behavior involves parasynaptic OT release from axon terminals that originate in the PVN and make synaptic contact with OTR-expressing dendrites which do not undergo estradiol-induced retraction. Concomitantly, dendrites that do not express OTR retreat in the presence of estradiol

via a yet undefined mechanism. As a result, boutons that were in contact with the now-retracted dendrites reorganize to innervate the remaining OTR+ dendrites in order to increase glutamate neurotransmission and dampen sexual behavior. The addition of progesterone reverses the reconfiguration in order to facilitate lordosis. While many details of this circuit remain unclear, we have demonstrated key details of OT in the LFC.

Here I described the ovarian hormone-regulated synaptic reorganization in the LFC, an area important for afferent input and behavioral modulation as well as the localization and significance of the neuromodulator OT. These effects are related to the estradiol-induced retraction of vlVMH dendrites from the LFC, one of the main proposed mechanisms of lordosis regulation. In the next chapter, I address mechanisms and a timecourse related to another main effect of hormone-induced plasticity, spinogenesis, which likely controls local connectivity within the nucleus. Specifically I analyzed the expression of glutamate ionotropic AMPA receptor subunits GluA1 and GluA2, and the actin- and dendritic spine-associated protein cofilin at various timepoints after hormone administration.

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Author Contirbutions

Journal of Comparative Neurology 518:4531-4545: LMF, EJV, and GDG designed research; BAR and GDG performed research; GDG and SLF analyzed data; GDG and LMF wrote paper.

Hormones and Behavior 61:251-258: LMF and SLF designed research; SLF analyzed data; LMF and SLF wrote paper.

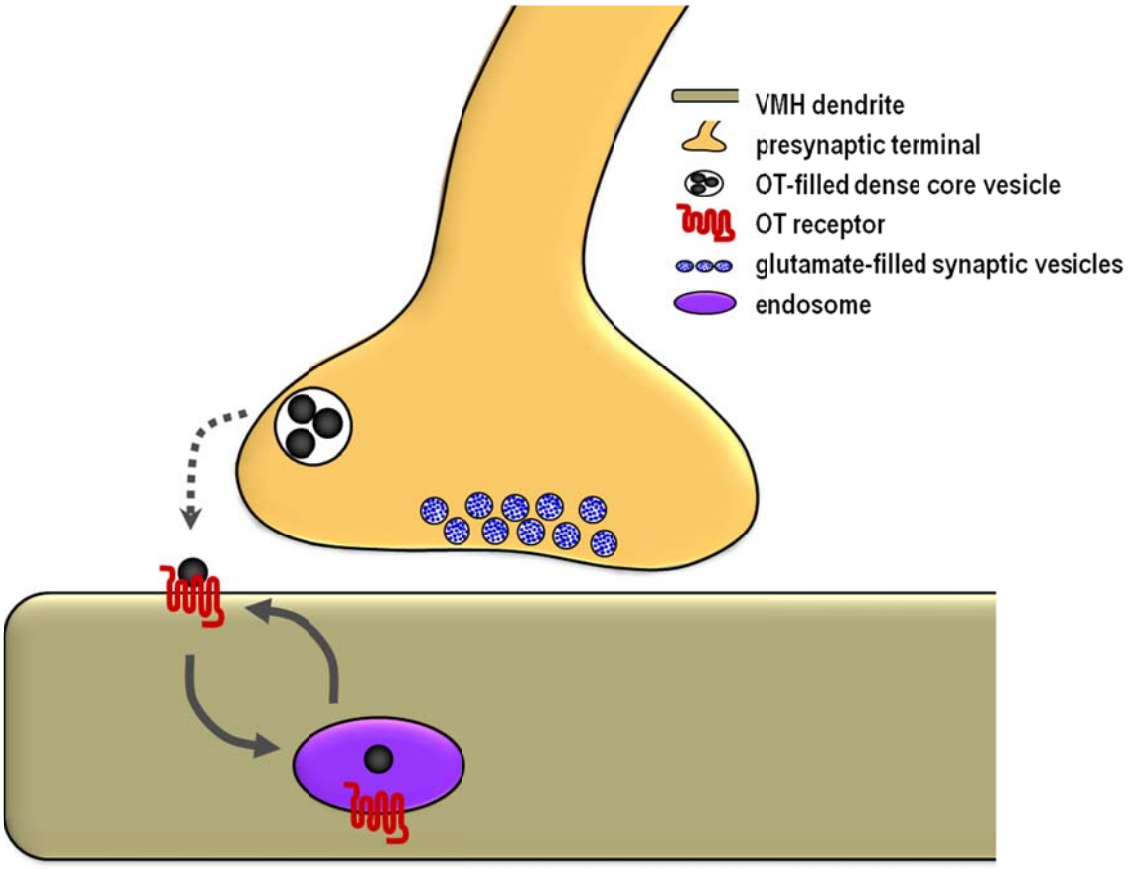

Figure 3.7. A schematic illustrating the hypothesized parasynaptic release of OT from glutamate terminals, and its receptor binding and internalization into VMH dendrites.

CHAPTER 4: Ovarian hormones regulate phospho-cofilin and AMPAR subunits GluA1 and GluA2 in the hypothalamic ventromedial nucleus and amygdala of female rats

Abstract

Estradiol modulates dendritic spines in the ventrolateral portion of the hypothalamic ventromedial nucleus (vlVMH) in a spatially specific manner that likely contributes to the control of female sexual receptivity. Previous studies have identified the phosphorylation of the actin-associated protein cofilin as a necessary step in spinogenesis. Additionally, ionotropic AMPA glutamate receptor subunits GluA1 and GluA2 have been associated with spine morphology and neuroplasticity. The present study investigates the expression of the ratio of phosphorylated to total cofilin, GluA1, and GluA2 using immunohistochemistry. We examined several subnuclei of the hypothalamus and amygdala at several timepoints after administration of ovarian hormones, as these areas have been associated with ovarian hormone-regulated behaviors such as lordosis and anxiety, respectively. We observed a rapid, transient estradiol-mediated increase in the ratio of p-cofilin:cofilin within 30 minutes in the vlVMH and adjacent lateral fiber complex (LFC), which is likely the result of membrane receptor action. We also identified slower changes in GluA1, which may reflect genomic estrogen receptor action. Therefore, there are dynamic and spatially-specific changes in protein expression in these brain areas over a wide timecourse that serve as a first step in determining the mechanism underlying ovarian hormone-induced spine changes in the vlVMH and how they relate to the lordosis circuit.

Introduction

Estradiol induces neuroplasticity in many brain areas in response to numerous conditions and stimuli (Griffin and Flanagan-Cato, 2011; Flanagan-Cato, 2011; Cooke and Woolley, 2005; Micevych and Christensen, 2012; Rasia-Filho et al., 2012). One important neuronal element regulated by estradiol is expression of dendritic spines, the major site of excitatory input in the brain (Lee et al., 2012). The most well defined factors of estradiol-induced spinogenesis have been gleaned from studies in the hippocampus, and to a lesser extent, the hypothalamus. These hippocampal and hypothalamic structural changes are associated with essential behaviors such as spatial memory function and reproduction, respectively. While a number of studies have been devoted to identifying components of the molecular cascades and mechanistic timing, many details remain ambiguous.

Spines of various shapes and sizes have been discovered on numerous cell types throughout the brains of all vertebrates and some invertebrates (Rochefort and Konnerth, 2012). Dendritic spines display a variety of profiles and states that can affect their function, including variable synapse strength and density of ionotropic glutamate receptor expression (Rochefort and Konnerth, 2012; Lee et al., 2012). As previously mentioned, spines have been associated with multiple behaviors, including female sexual behavior. In the ventrolateral subdivision of the ventromedial nucleus of the hypothalamus (vlVMH), ovarian hormones regulate spine density, likely in order to influence reproductive behavior. This hormone-mediated spinogenesis has been demonstrated with exogenous estradiol administration to ovariectomized (OVX) female rats as well as in naturally cycling females. In both cases, higher levels of estradiol are correlated with increased dendritic spine density (Frankfurt et al., 1990; Madeira et al., 2001). However, hormones have more complex effects than global spine growth. Specifically, in response to estradiol treatment, spine density is increased on short primary dendrites and decreased on long primary dendrites of neurons that project to the periaqueductal gray (PAG), which are critical to the display of lordosis (Calizo and Flanagan-Cato, 2000; Calizo and Flanagan-Cato, 2002; Sakuma and Pfaff, 1979; Hennessey et al., 1990). Importantly, these effects are specific to

dendrites of neurons within the ventrolateral, but not dorsomedial, portion of the VMH (Calizo and Flanagan-Cato, 2000; Calizo and Flanagan-Cato, 2002; Madeira et al., 2001). This demonstrates a spatial specificity that almost certainly contributes to the neurochemical and anatomical features of the nucleus that are necessary for lordosis behavior.

Despite such specific characterization of estradiol-induced spinogenesis in the vlVMH, the mechanism and associated proteins involved have yet to be determined. One likely component is the protein cofilin, which disassembles actin filaments. Phosphorylation of cofilin deactivates it, allowing actin to polymerize. Importantly, phosphorylation of cofilin (p-cofilin) is necessary for dendritic spine formation (Kramar et al., 2009; Rochefort and Konnerth, 2012). In a series of experiments utilizing hippocampal slices, Kramar et al. (2009, 2012) describe a cascade of events in which estradiol increases p-cofilin via stimulation of the small GTPase RhoA, which activates RhoA kinase (ROCK), resulting in phosphorylation of LIM kinase, which in turn phosphorylates cofilin, ending in an increase in F-actin in spines. Cofilin has been implicated in estradiol-induced spinogenesis in hypothalamic nuclei as well. In the arcuate nucleus, metabotropic glutamate receptor mGluR1a activity is necessary for an estradiol-induced increase in p-cofilin, which was associated with increased dendritic spine density. This spinogenesis was indicated to be necessary for lordosis behavior because a toxin that disrupts β–actin levels also attenuates lordosis (Christensen et al., 2011). However, it is not known if estradiol increases β-actin or if the toxin disrupts spinogenesis. Therefore, it is unclear whether similar mechanisms are involved in the vlVMH, how spinogenesis in that area relates to reproductive behavior, the timecourse of changes, and progesterone's effects, if any.

Other possible contributors to estradiol-induced spinogenesis in the vlVMH are ionotropic glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR). Specifically, AMPAR subunits GluA1 and GluA2 have been associated with synaptic plasticity in many brain areas. In hippocampal cells, GluA2 overexpression increases spine number, length, and width, and induces spinogenesis on previously nonspiny GABA interneurons (Passafaro et al., 2003). Likewise, AMPAR antagonists and GluA2-siRNA, which interferes with GluA2

expression, decrease spine formation in hippocampal slice cultures and GluA2 knockout mice display altered synaptic patterns (Passafaro et al., 2003; McKinney et al., 1999; Richards et al., 2005; Medvedev et al., 2008). Finally, AMPAR activity is necessary for estradiol-induced spinogenesis in the mediobasal hypothalamus during development (Schwarz et al., 2008). Because there is evidence that AMPAR may be involved in dendritic spine density changes in the vlVMH, it is important to note that GluA1 and GluA2/3 mRNA and protein expression have been demonstrated in the hypothalamus of male and female rats (Diano et al., 1997; Eyigor et al., 2001). Expression of both subunits increases with estradiol treatment in both sexes, but GluA2/3 receptors are amplified significantly more in the female versus male hypothalamus (Diano et al., 1997). Importantly, the effect of the addition of progesterone, which maximizes the lordosis response, has not been observed, nor have the effects of physiological levels of estradiol in the VMH specifically been described (Diano et al., 1997). If AMPAR are involved in hormone-induced spinogenesis, they may be necessary for cofilin phosphorylation, or they may be a result of p-cofilin, inserted into newly formed spines.

Finally, the timecourse of spine induction in the vlVMH has yet to be described. The increase in spine density is observed at 72-76 hours post-estradiol treatment, or late in the estrous cycle, when the animal would display sexual receptivity, but the spines and molecules involved in estradiol-induced spinogenesis may be present or active earlier (Frankfurt et al., 1990; Segarra and McEwen, 1991; Calizo and Flanagan-Cato, 2000; Christensen et al., 2011). Numerous studies cite rapid estradiol action in various brain areas as well as peripherally, and many of these early effects have been proven to be the result of membrane-associated receptors (see Vasudevan and Pfaff, 2008 for review). Specifically, in the VMH, electrophysiological effects of estrogen are apparent in three to five minutes (Minami et al., 1990). Also within the VMH, estradiol increased phosphorylated cAMP response element-binding protein (pCREB) immunoreactivity in one hour (Abraham and Herbison, 2005). Less rapid estradiol effects related to spine formation have been observed *in vivo* in other hypothalamic areas. During development, spinogenesis is apparent in the mediobasal hypothalamus in six hours, while it is observed in the

adult female arcuate nucleus within four hours (Schwarz et al., 2008; Christensen et al., 2011). In the latter case, increased p-cofilin labeling necessary for spinogenesis was observed within one hour of estradiol treatment (Christensen et al., 2011). The rapidity in which estradiol is able to enact this change is suggestive of non-genomic, membrane-associated actions (see Vasudevan and Pfaff, 2008 for review). Therefore, identifying details about the timing and elements involved in spine induction in the vlVMH over the estrous cycle may shed light on mechanisms that underlie estradiol-induced plasticity as well as the components necessary for reproduction. Here we test the hypotheses that 1) the phosphorylated form of the protein cofilin is present in the vlVMH and is rapidly upregulated by ovarian hormones, which likely contributes to estradiol-induced spinogenesis and 2) ovarian hormones downregulate GLuA1 expression at a later timepoint, as glutamate is inhibitory to lordosis behavior. These results will serve as a first step towards indication of membrane estrogen receptor involvement in estradiol-induced spinogenesis in the vlVMH and its importance in the lordosis circuit.

Materials and methods

Animals

Adult female Sprague-Dawley rats weighing 220-250 g were obtained from Taconic (Hudson, NY) and group-housed in plastic tubs (41 x 21 x 22 cm) with standard bedding. Rat chow and water was available *ad libitum*. The colony was maintained at 22°C on a 12/12-hour reverse light/dark cycle, with lights off at 1000 hours. Animals were allowed one week to acclimate before any procedures were performed. The Institutional Animal Care and Use Committee of the University of Pennsylvania approved all animal procedures.

Ovariectomy

Surgeries were performed under aseptic conditions. Animals were bilaterally ovariectomized (OVX) using aseptic technique during general anesthesia: 90 mg/kg ketamine

and 9 mg/kg xylazine (both intraperitoneally, and both Phoenix Pharmaceutical, Inc., St. Joseph, MO). The bilateral lumbar incisions were closed with absorbable chromic gut sutures and suture clips (Fine Science Tools, Foster City, CA). After surgery, rats were given yohimbine (2.1 mg/kg, ip, Lloyd Laboratories, Shenandoah, IA) to counteract the anesthesia produced by xylazine. Animals were given one week of monitored recovery before experiments commenced.

Intracerebroventricular Cannulation

Surgeries were performed under aseptic conditions. OVX females were anesthetized with isofluorane gas. A 26-gauge stainless steel cannula (Plastics One, Roanoke, VA) was unilaterally, stereotaxically implanted into the lateral ventricle (0.48 mm caudal to bregma, 1.6 mm from the midline, and 4.2 mm ventral to dura mater) were affixed to the skull with dental cement and three 0-80 thread, 1/8-inch stainless steel mounting screws (Plastics One). Left and right ventricles were alternated. Wound clips were used to close the incision around cannula. Guide cannulae were maintained with 33-guage dummy cannulae (Plastics One) except when infusions were taking place.

Animals were allowed at least five days to recover before verification procedures were performed. To test for correct cannula placement, animals were given an icv injection of 20 ng of Angiotensin II diluted in 2 µl aCSF (Harvard Apparatus, Holliston, MA) via a Hamilton syringe connected with PE-10 tubing to a 33-guage injection cannula that terminated 1 mm beyond the guide cannula. Animals that failed to drink vigorously within 30 seconds of infusion were used under control conditions only.

After three additional days of recovery, animals were infused with 3 µl aCSF, 100 mM DL-2-amino-5-phosphonopentanoic acid (NMDAR antagonist; AP-5) (Sigma-Aldrich, St. Louis, MO) in 3 µl aCSF, or 4.3 mM CNQX (AMPA/kainite glutamate receptor antagonist) (Sigma-Aldrich, St. Louis, MO) in 3 µl aCSF. All animals were injected sc with vehicle (veh) or estradiol benzoate (EB) 15 minutes later (see below). They were perfused 30 minutes after injection (see below). In summary, the experimental design comprised the following groups: vehicle/vehicle,

EB/vehicle, EB/AP-5, EB/CNQX. Infusions were given at the beginning of the dark cycle over 60 seconds but internal cannulae were left in place for an additional two minutes. Different tubing and internal cannulae were used for each solution.

Hormone Treatment

One week after surgery, one set of animals was randomly assigned to one of three hormone treatment groups: vehicle (veh), estradiol benzoate (EB) alone, or EB combined with progesterone (EBP) on a four-day cycle shown to induce lordosis. The veh control group received injections of sesame oil (100 µl, sc) on days one and two, followed by propylene glycol (100 µl, sc) on day four. The two other treatment groups were primed with 17β-estradiol benzoate (EB; 10 µg in 100 µl sesame oil, sc, Sigma, St. Louis, MO) on days one and two. On day four, the EB group received vehicle (propylene glycol; 100 µl, sc) and the EBP group, progesterone (500 µg in 100 µl propylene glycol, sc, Sigma). Four hours after the injection on day four, animals were perfused (see below).

Another set of OVX females was given a single injection of veh or EB and perfused 30 minutes, one, or four hours later.

Enzyme linked immunosorbent assay (ELISA)

Thirty or 60 minutes after animals were injected sc with vehicle or EB (see above), 2 mm was cut from the tip of the tail to obtain blood, which was collected in Microvette CB 300 Z capillary collection tubes (Sarstedt, Newton, NC). Tubes were centrifuged at 4º C for 15 minutes to separate serum. Duplicates of 25 µl of serum were run according to the Calbiotech estradiol ELISA kit.

Perfusion and Sectioning

Animals were anesthetized (90 mg/kg ketamine and 9 mg/kg xylazine, intraperitoneal) and perfused transcardially through the ascending aorta with 100 ml saline followed by 200 ml

4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA). The brains were isolated, post-fixed in paraformaldehyde overnight at 4°C, and then submerged in 30% sucrose in 0.1 M phosphate buffer. Coronal sections encompassing the VMH were cut on a freezing microtome (Olympus, Tokyo, Japan) into four serial sets of 40-µm-thick slices. Sections were stored in cryoprotectant at -20°C.

Immunohistochemistry

Sections were washed in Tris-buffered saline (TBS; pH 7.4), 0.3% hydrogen peroxide (H_2O_2, Fisher Scientific; Fair Lawn, NJ), and TBS again. They were then incubated with Anti-Glutamate Receptor 2 antibody (1:200, AB1768, rabbit, Millipore, Temecula, CA), Anti-Glutamate Receptor 1 antibody (1:150, AB1504, rabbit, Millipore, Temecula, CA), anti-phospho-cofilin antibody (1:100, rabbit, Cell Signaling, Danvers, MA), or anti-cofilin antibody (1:100, rabbit, Cell Signaling, Danvers, MA) in TBS with 0.2% TritonX-100 and 3% normal goat serum (Jackson ImmunoResearch; West Grove, PA) for 2 days at 4°C. After several TBS washes, sections were incubated in Biotin-SP-AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch) in TBS with 0.2% TritonX-100 and 3% normal goat serum (Jackson ImmunoResearch) for 2.5 h at room temperature. Following several TBS washes, sections were incubated in avidin-biotin-peroxidase complex (Elite Standard ABC kit, Vector Laboratories, Burlingame, CA) for 1.5 h. Sections were then treated with 50 mM Tris containing 3,3-diaminobenzidine (DAB, 0.2 mg/ml, Fisher Scientific; Fair Lawn, NJ), nickel sulfate (25 mg/ml, Sigma-Aldrich, St. Louis, MO), and 0.025% H_2O_2 for 25 min. Finally, sections were washed in TBS, mounted on gel-coated slides, air-dried, and coverslipped with Permount mounting media (Fisher Scientific).

An additional group of sections was washed in TBS then incubated with 1:100 Anti-Glutamate Receptor 1 antibody (see above) and ERα antibody (1:50, 1D5, sc-56833, mouse, Santa Cruz Biotechnology, Dallas, TX) in TBS with 0.2% Triton X-100 and 3% normal goat serum for 2 days at 4°C. After several TBS washes, sections were incubated in cy3 conjugated goat anti-mouse antiserum (1:100, Jackson ImmunoResearch) and DyLight 488 goat anti-rabbit IgG

(1:100, Jackson ImmunoResearch) in TBS with 0.2% Triton X-100 and 3% normal goat serum (Jackson ImmunoResearch) for 2.5 h at room temperature. After several washes, sections were mounted on slides, air-dried, and coverslipped with DPX mounting media (Electron Microscopy Sciences).

Immunohistochemical Analysis

Images were acquired using a digital camera (Diagnostic Instruments, Sterling Heights, MI, model RTKE) with an Olympus Optical BX50 microscope (Olympus, Tokyo, Japan). Images were then analyzed by an experimenter blind to the treatment groups. A standardized shape encompassing the vlVMH, dmVMH, LFC, BLA, or CeA, as identified by a rat brain atlas (Paxinos and Watson, 2005), was placed over the region of interest and optical density was measured using the public domain program ImageJ (http://rsbweb.nih.gov/ij/). Because the CeA changes shape over the rostral-caudal progression, two circles were used to measure optical density in this brain region; a larger one in more rostral regions and a smaller one more caudally. Optical density of a background circle encompassing an area devoid of staining in the optic tract was subtracted from the area value. For each animal, the quantification reflected the average values from three to ten 40-µm sections.

Golgi impregnation and morphological analysis

OVX females were treated with veh or EB and sacrificed at four or 76 hours (see above). Animals were anesthetized (90 mg/kg ketamine and 9 mg/kg xylazine, intraperitoneal) and brains were quickly removed and prepared according to the FD Rapid GolgiStain™ Kit User Manual (FD NeuroTechnologies; Ellicot City, MD). Brains were incubated in a potassium dichromate, mercuric chloride, and potassium chromate solution for 2 weeks. After this incubation, VMH sections (200 µm) were obtained using a vibratome (Vibratome® Series 1000). Sections then were placed onto gelatin-coated slides, stained with cresyl violet, dehydrated in ethanol, and coverslipped using Permount® (Fisher Scientific).

Sections containing the VMH were viewed with a BX50 microscope (Olympus; Central Valley, PA). Neurons were considered to be in the ventrolateral VMH (vlVMH) if they were located within the borders of the subdivision according to clear demarcations visible with the Cresyl violet staining. vlVMH neurons then were visualized at 1000X and traced using camera lucida. Next, the tracings were scanned using a Hewlett-Packard Scanjet 3970 and dendrite length was measured using ImageJ. Only neurons in which the entire dendritic arbor could be visualized within a single 200-μm section were analyzed. All morphological analyses were performed with the experimental group concealed.

Dendrites were categorized as in previous studies (Calizo and Flanagan-Cato, 2000 and Calizo and Flanagan-Cato, 2002). We analyzed a minimum of five neurons per animal, each with clearly visible dendrites. Dendrite length for each dendrite type was calculated based on an average of triplicate measurements. The dendrite length for the short primary dendrites was averaged when there was more than one of this dendrite type on a given neuron. Dendritic spine density was analyzed as a function of number of spines per dendrite length, was also counted in triplicate and averaged. Values for each animal were calculated as an average of the values per neuron. The *n* presented in the results reflects the number of animals per group.

Statistical Analysis

Data was analyzed by student's t-test, one-, or two-way ANOVA by brain area, with Newman-Keuls or Bonferroni as the post hoc test. Data are expressed as the mean ± standard error. All statistical comparisons were performed using the Prism 4.0 statistical software (GraphPad; San Diego, CA). Values are considered significant at $P<0.05$.

Results

Late hormone effects-cofilin in the VMH

Because changes in dendritic spine density in the vlVMH are present 76 hours after estradiol treatment (Frankfurt et al., 1990; Madeira et al., 2005; Calizo and Flanagan-Cato, 2000; Calizo and Flanagan-Cato, 2002), we assessed levels of immunoreactivity of molecules implicated in the formation of spines at the 76 hour timepoint (Kramar et al., 2009 and 2012; Rochefort and Konnerth, 2012; Christensen et al., 2011; Passafaro et al., 2003; McKinney et al.,, 1995l; Richards et al., 2005; Medvedev et al., 2008; Schwarz et al., 2008). Using immunohistochemistry, we assessed the actin-associated protein cofilin, its phosphorylated form (p-cofilin), and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) subuntis GluA1 and GluA2. Fig. 4.1A shows the boxes placed around the areas analyzed to determine the optical density of antibody staining (arbitrary units; AU). The dorsomedial and ventrolateral portions of the VMH (dmVMH and vlVMH, respectively) were assessed, as well as the lateral fiber complex (LFC), adjacent to the vlVMH. The vlVMH, but not the dmVMH, is associated with female sexual behavior. The LFC, which contains dendrites extending from the vlVMH that exhibit estradiol-induced plasticity, has been suggested to provide input necessary for lordosis behavior (Griffin et al., 2010). In all cases, a background area devoid of staining in the optic tract (OT; Fig. 4.1A) was subtracted from the region of interest value.

Fig. 4.1B illustrates a magnified representative photomicrograph of p-cofilin levels in the vlVMH. Staining was punctate in nature, but did not appear nuclear. Fig. 4.2 and 4.3 demonstrate representative hypothalamic p-cofilin and cofilin immunoreactivity, respectively, from females treated with vehicle (veh), estradiol benzoate (EB), and estradiol benzoate plus progesterone (EBP). Figures 4.4A-C, 4.5A-C, and 4.6A-C show quantification of the levels of p-cofilin, cofilin, and the ratio of p-cofilin to total cofilin in the vlVMH, dmVMH, and LFC, respectively. There were no significant differences in any of these measures for any of the VMH

areas surveyed; however, there was a trend for estradiol to decrease cofilin staining in the LFC compared to veh (0.193 ± 0.023 versus 0.237 ± 0.006 AU, $p < 0.090$, Fig. 4.6B).

Late hormone effects-cofilin in the amygdala

In order to compare whether possible changes in protein levels are global, we included a parallel analysis of the basolateral (BLA) and central (CeA) nuclei of the amygdala, illustrated in figure 4.7A. Representative p-cofilin staining in the amygdala is shown in figure 4.7B. As exemplified in Fig. 4.8, 4.9, 4.10A-C, and 4.11A-C, ovarian hormones did not affect the density of p-cofilin, cofilin, or the ratio of the former to latter.

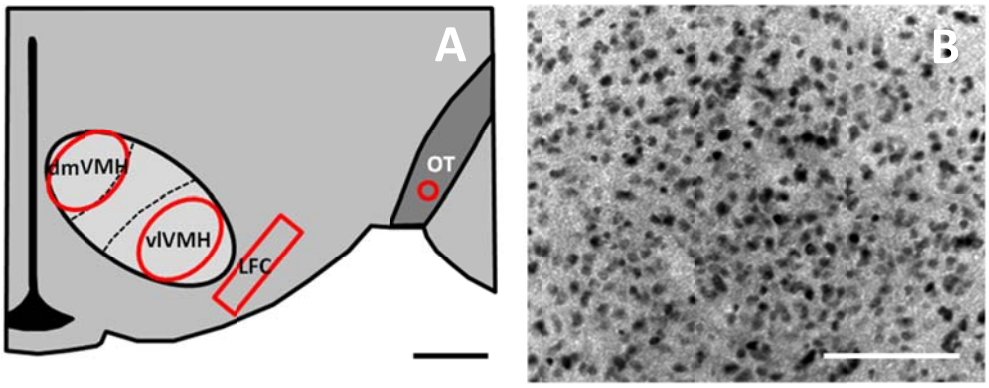

Figure 4.1. Immunohistochemical analysis area of VMH and p-cofilin labeling.
A. Representative placement of standardized shapes used to measure the ventrolateral subdivision of the ventromedial nucleus of the hypothalamus (vlVMH), dorsomedial subdivision of the VMH (dmVMH), lateral fiber complex (LFC), and optic tract (OT), which served as the subtracted background value, as little staining was present there. Bar = 500 µm. B. Magnified photomicrograph of p-cofilin immunoreactivity in the vlVMH. Bar = 100 µm.

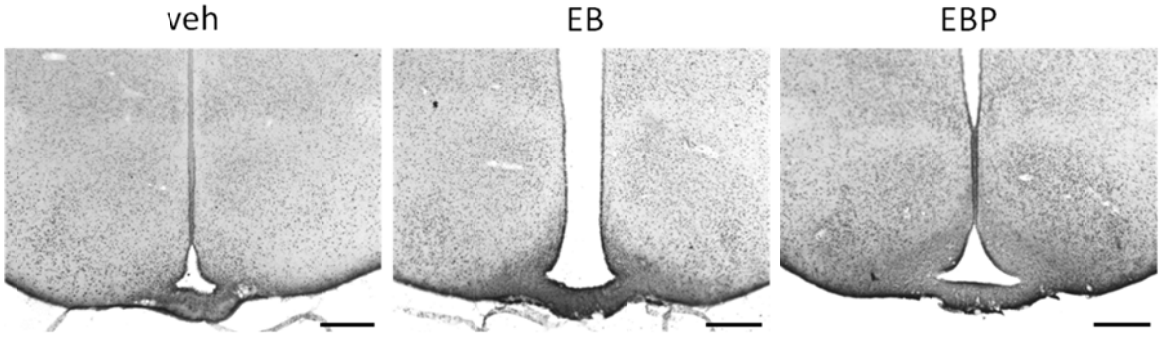

Figure 4.2. Photomicrographs of p-cofilin immunoreactivity in the VMH of rats treated with vehicle (veh), estradiol benzoate (EB), or EB plus progesterone (EBP). Protein levels were similar across groups. Bars = 500 µm.

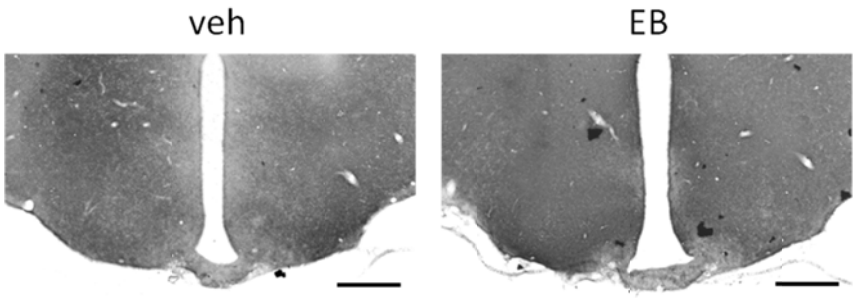

Figure 4.3. Photomicrographs of cofilin immunoreactivity in the VMH of rats treated with vehicle (veh) or estradiol benzoate (EB). Protein levels were similar across groups. Bars = 500 µm.

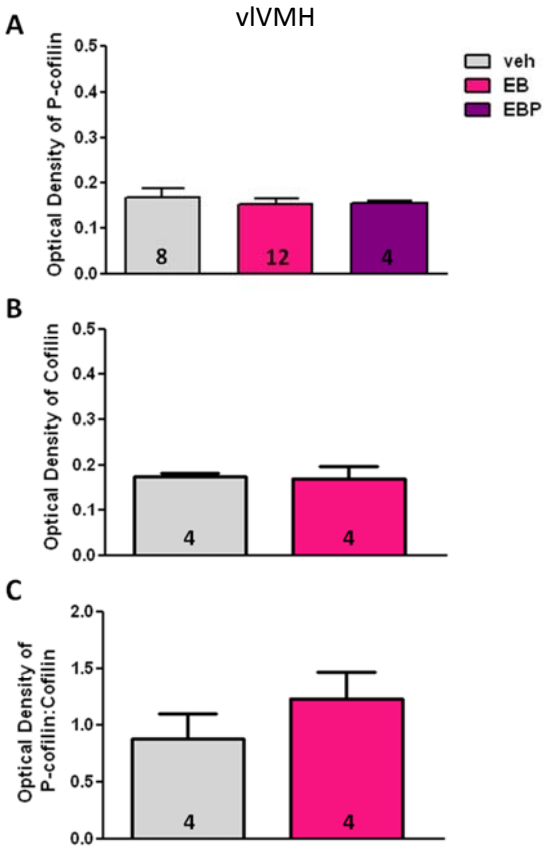

Figure 4.4. Quantification of protein levels in the vlVMH. No differences were observed in any of the measures. A. Optical density of p-cofilin. B. Optical density of cofilin. C. Optical density of the ratio of phosphorylated to total cofilin. Numbers in bars represent sample size.

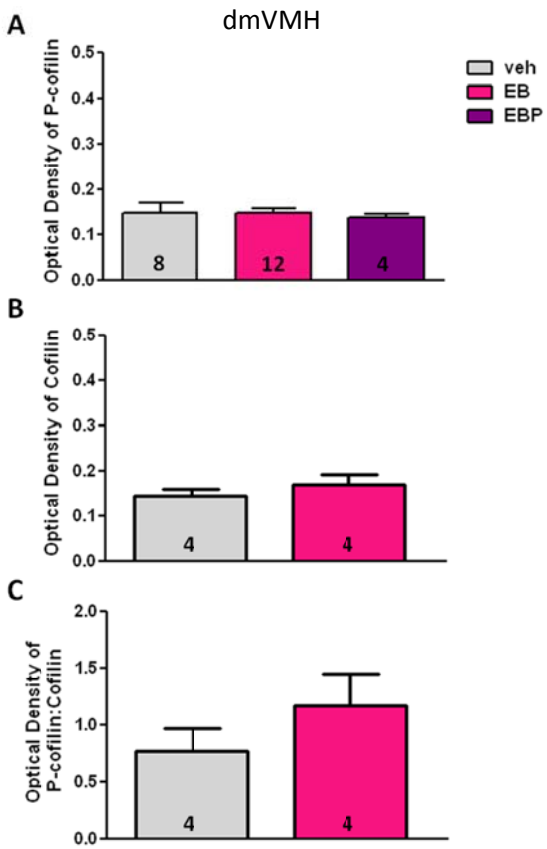

Figure 4.5. Quantification of protein levels in the dmVMH. No differences were observed in any of the measures. A. Optical density of p-cofilin. B. Optical density of cofilin. C. Optical density of the ratio of phosphorylated to total cofilin. Numbers in bars represent sample size.

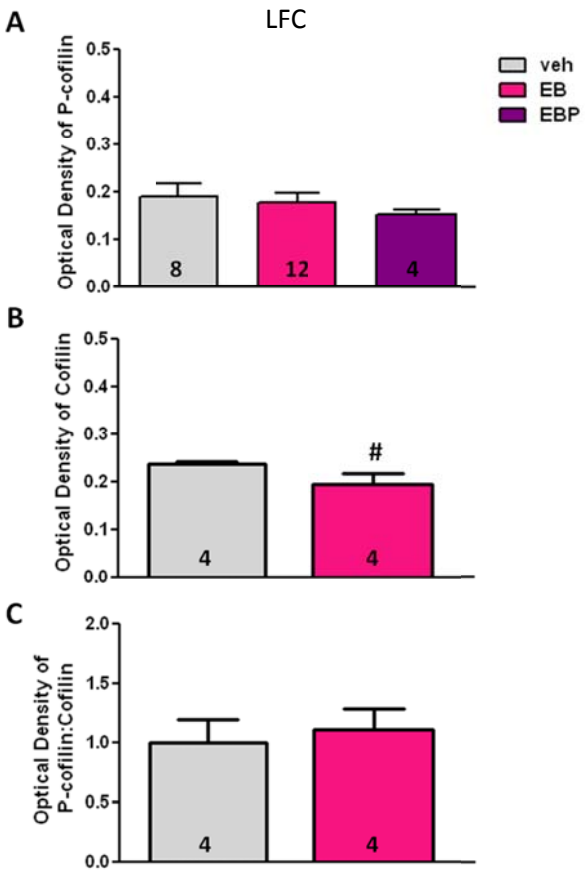

Figure 4.6. Quantification of protein levels in the LFC.

A. Optical density of p-cofilin. There were no differences across treatment groups.

B. Optical density of cofilin. There was a trend for estradiol to decrease cofilin in the LFC. #, p< 0.10

C. Optical density of the ratio of phosphorylated to total cofilin. There were no differences across hormone treatment groups.

Numbers in bars represent sample size.

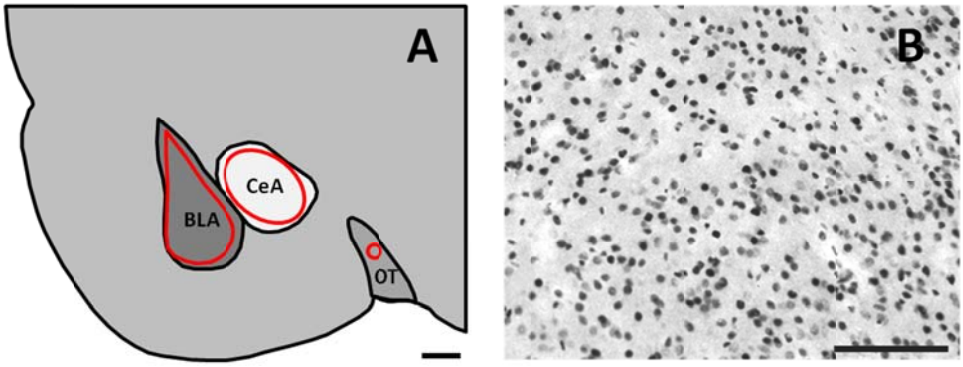

Figure 4.7. Immunohistochemical analysis area of the amygdala and p-cofilin labeling. A. Representative placement of standardized shapes used to measure the basolateral amygdaloid nucleus (BLA), central amygdaloid nucleus (CeA), and optic tract (OT), which served as the subtracted background value as little staining was present there. Bar = 500 µm. B. Magnified photomicrograph of p-cofilin immunoreactivity in the amygdala. Bar = 100 µm.

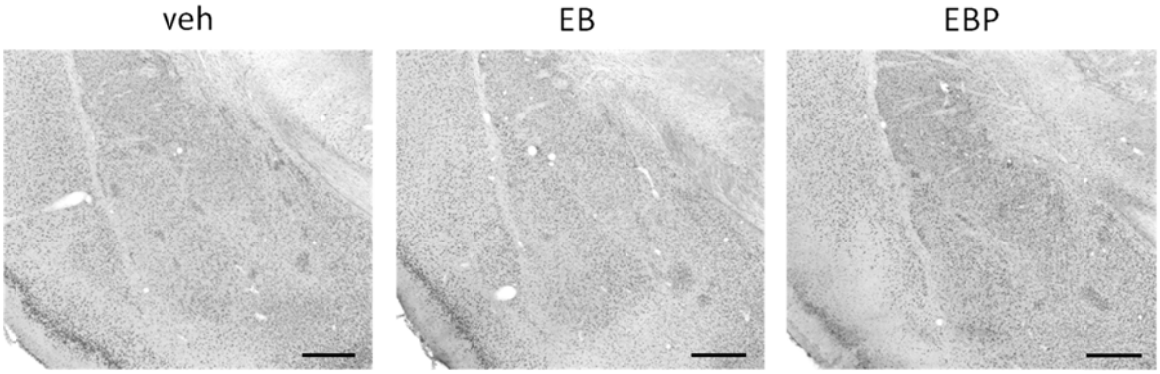

Figure 4.8. Photomicrographs of p-cofilin immunoreactivity in the BLA and CeA of rats treated with vehicle (veh), estradiol benzoate (EB), or EB plus progesterone (EBP). Protein levels were similar across groups. Bars = 500 µm.

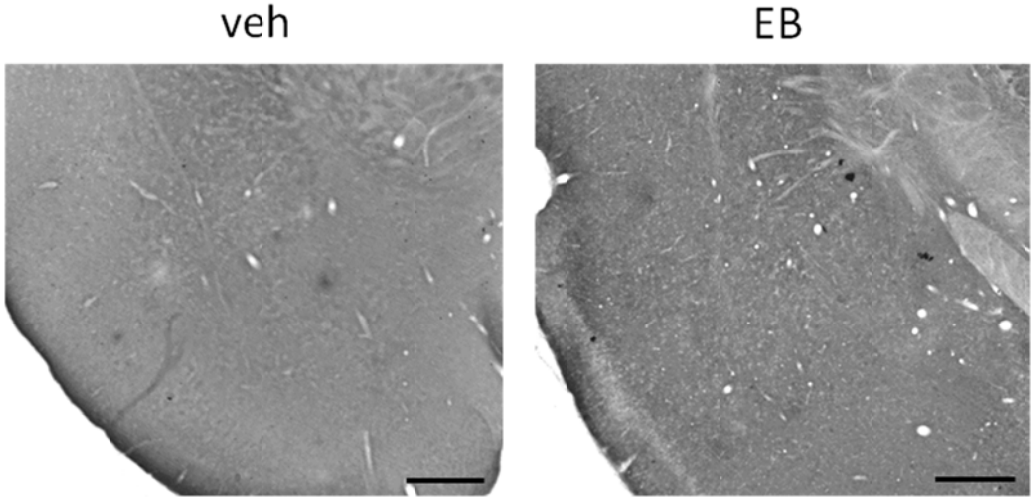

Figure 4.9. Photomicrographs of cofilin immunoreactivity in the BLA and CeA of rats treated with vehicle (veh) or estradiol benzoate (EB). Protein levels were similar between groups. Bars = 500 µm.

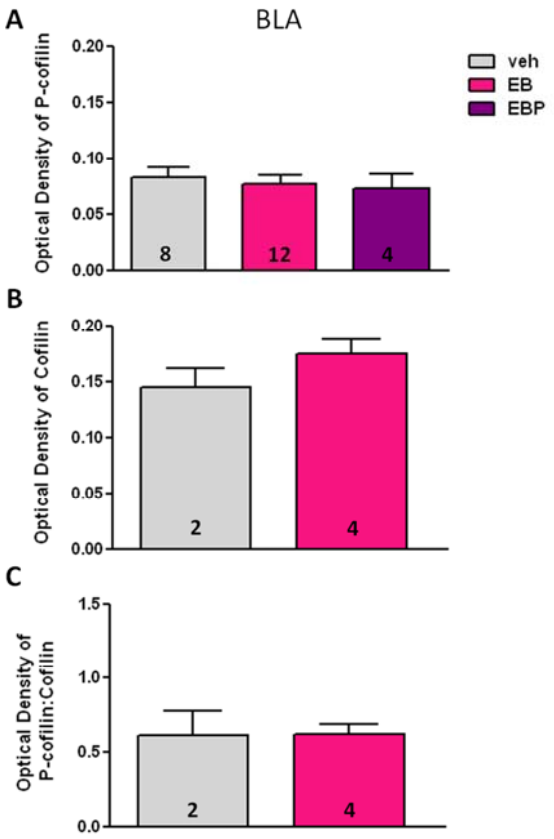

Figure 4.10. Quantification of protein levels in the BLA. No differences were observed in any of the measures. A. Optical density of p-cofilin. B. Optical density of cofilin. C. Optical density of the ratio of phosphorylated to total cofilin. Numbers in bars represent sample size.

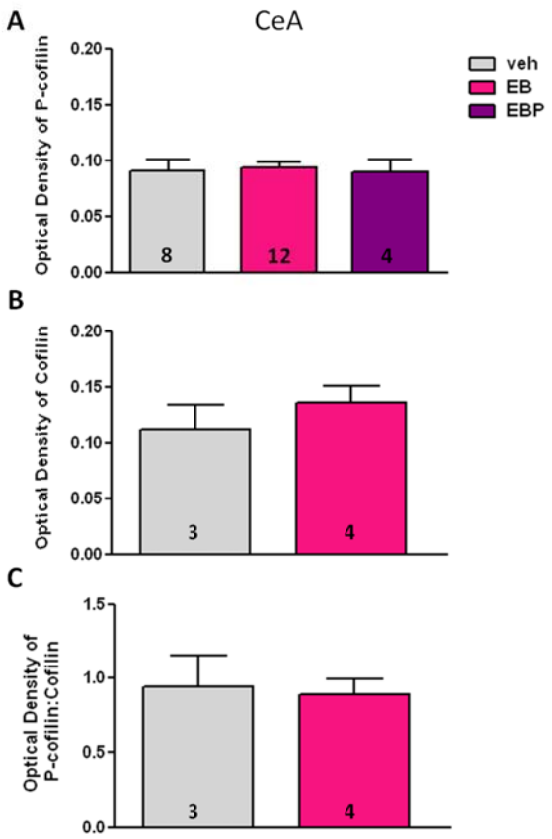

Figure 4.11. Quantification of protein levels in the CeA. No differences were observed in any of the measures. A. Optical density of p-cofilin. B. Optical density of cofilin. C. Optical density of the ratio of phosphorylated to total cofilin. Numbers in bars represent sample size.

Late hormone effects-AMPAR subunits in the VMH

Although there were no differences in cofilin levels or phosphorylation at the later timepoints, there were some changes in AMPAR subunits. The magnified immunoreactivity of GluA1 and GluA2 is pictured in Figure 4.12A and 4.12B, respectively. GluA1 immunoreactivity appears fibrous while GluA2 is visible in the cytoplasm and weakly in dendrites. GluA1 immunoreactivity at 76 hours post-treatment in the veh, EB, and EBP conditions is represented in figure 4.13. Expression extending out of the boundaries of the VMH nucleus into the LFC is apparent in all groups. The level of staining in the vlVMH, dmVMH, and LFC is quantified in Fig.

4.14A-C. Ovarian hormones had a significant effect on GluA1 density in the vlVMH ($F_{(2,38)}$ = 5.197, p < 0.020, Fig. 4.14A) and LFC ($F_{(2,38)}$ = 3.28, p < 0.050, Fig. 4.14C), but not in the dmVMH (Fig. 4.14B). Specifically, in the vlVMH, estradiol plus progesterone significantly increased GluA1 density from the estradiol alone (0.393 ± 0.021 versus 0.337 ± 0.013 AU, p < 0.050) and the veh condition (0.393 ± 0.021 versus 0.313 ± 0.018 AU, p < 0.010, Fig. 4.14A), while in the LFC, estradiol plus progesterone increased GluA1 levels from vehicle-treated levels (0.361 ± 0.026 versus 0.287 ± 0.020 AU, p < 0.090, Fig. 4.14A). GluA2 immunoreactivity in the vlVMH also differed between treatment groups ($F_{(2,29)}$ = 5.903; p < 0.008, Fig. 4.15). In particular, estradiol administration reduced GluA2 from veh levels (0.087 ± 0.008 versus 0.119 AU ± 0.011, p < 0.050) and in comparison to estradiol plus progesterone levels (0.087 ± 0.008 versus 0.134 ± 0.012 AU, p < 0.010, Fig. 4.16A). There were no significant differences across groups in the dmVMH (Fig. 4.16B) and there was no appreciable GluA2 immunoreactivity present in the LFC in any treatment group (Fig. 4.15).

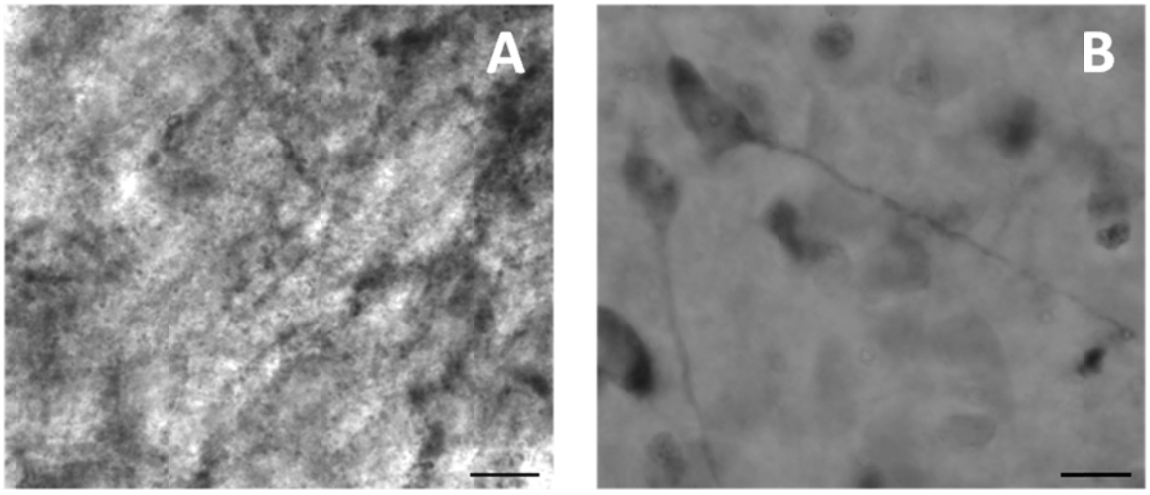

Figure 4.12. Representative AMPAR subunit immunoreactivity.
A. A magnified view of GluA1 immunoreactivity in the vlVMH. B. A magnified view of GluA2 immunoreactivity in the vlVMH. Bars = 10 µm.

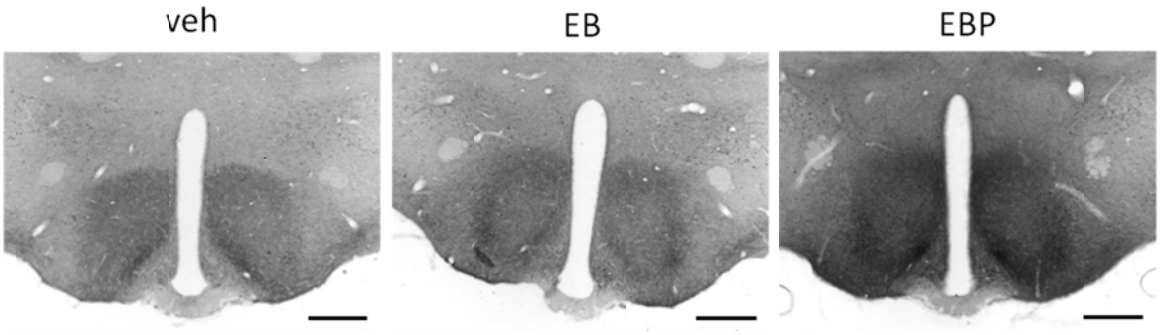

Figure 4.13. Photomicrographs of GluA1 immunoreactivity in the VMH of rats treated with vehicle (veh), estradiol benzoate (EB), or EB plus progesterone (EBP). Protein levels were significantly higher in the EBP condition in the vlVMH and LFC. Bars = 500 µm.

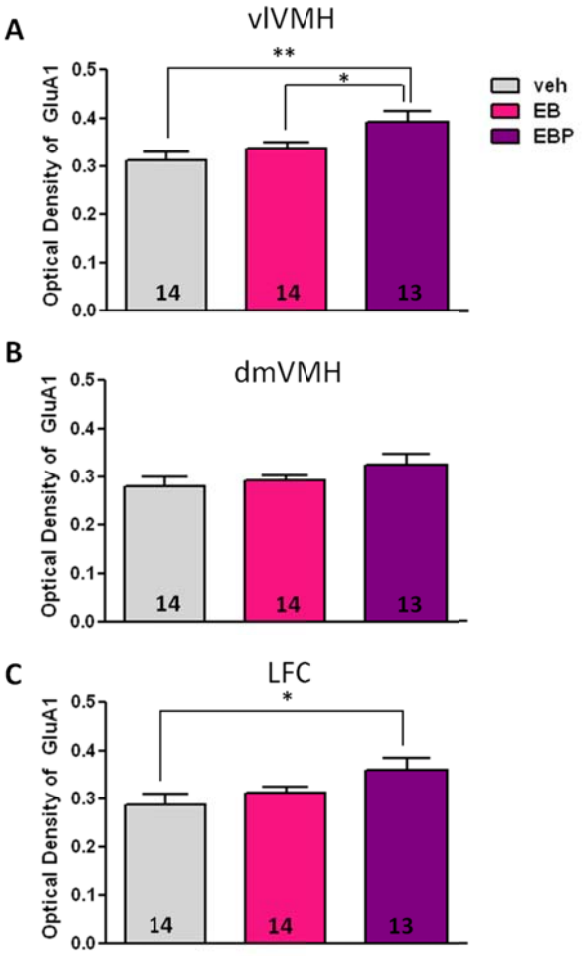

Figure 4.14. Quantification of GluA1 immunoreactivity.

A. Optical density of GluA1 in the vlVMH. Expression was significantly higher in the EBP condition. **, $p < 0.01$ compared to veh, *, $p < 0.05$ compared to EB.

B. Optical density of GluA1 in the dmVMH. No differences were present across treatment groups.

C. Optical density of GluA1 in the LFC. Expression was significantly increased in the EBP condition. *, $p < 0.05$ compared to the EB alone group.

Numbers in bars represent sample size.

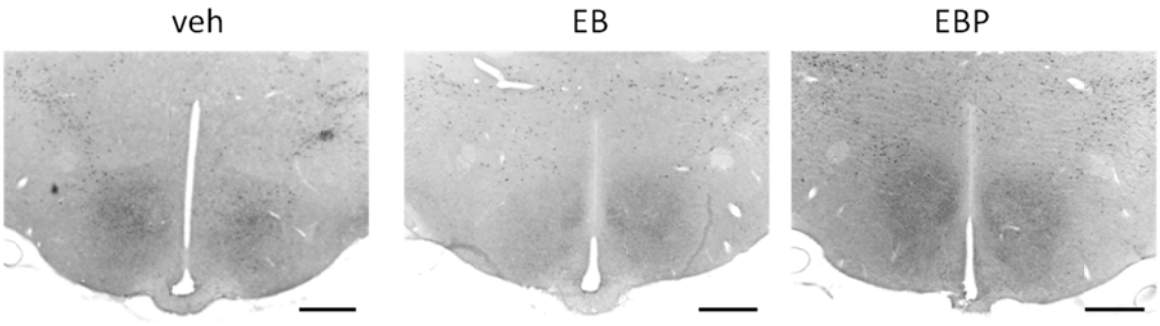

Figure 4.15. Photomicrographs of GluA2 immunoreactivity in the VMH of rats treated with vehicle (veh), estradiol benzoate (EB), or EB plus progesterone (EBP). Protein levels were significantly reduced in the EB condition in the vlVMH. Bars = 500 µm.

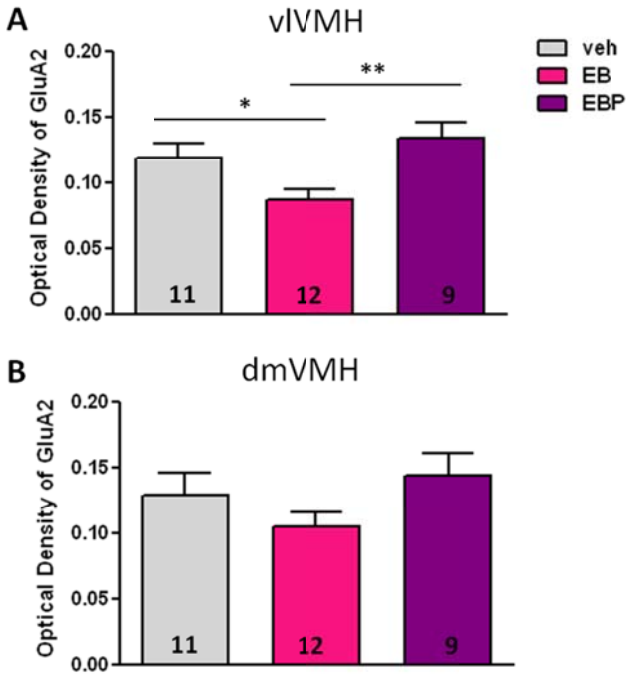

Figure 4.16. Quantification of GluA2 levels.

A. Optical density of GluA2 in the vlVMH. Expression was significantly reduced in the EB condition compared to that in the veh or EBP groups. *, $p < 0.05$ compared to veh, **, $p < 0.01$ compared to EBP.

B. Optical density of GluA2 in the dmVMH. No hormone effects on expression were observed.

Numbers in bars represent sample size.

Late hormone effects-AMPAR in the amygdala

In the amygdala 76 hours after ovarian hormone administrations, no changes in GluA1 levels were observed in either the BLA or CeA (Fig. 4.17 and 4.18A-B). However, as depicted in Fig. 4.19 and 4.20A-B, GluA2 immunoreactivity was ovarian hormone-sensitive ($F_{(2,29)} = 4.292$; $p < 0.030$). A post hoc test revealed that estradiol alone significantly decreased GluA2 compared to estradiol plus progesterone (0.218 ± 0.018 versus 0.324 ± 0.029 AU, $p < 0.050$, Fig. 4.20A), which was restored to vehicle levels. Similar patterns were present in the CeA as well

($F_{(2,29)}$ =5.249; $p < 0.020$). Estradiol significantly lowered levels of GluA2 when compared to estradiol plus progesterone (0.205 ± 0.015 versus 0.308 AU ± 0.024, $p < 0.010$) and there was a trend for estradiol to decrease GluA2 when compared to vehicle as well (0.205 ± 0.015 versus 0.266 ± 0.028 AU, $p < 0.060$, Fig. 4.20B).

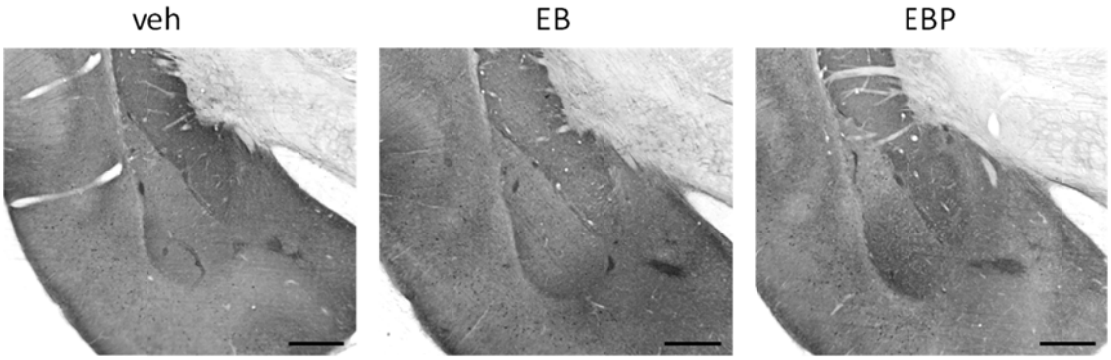

Figure 4.17. Photomicrographs of GluA1 immunoreactivity in the amygdala of rats treated with vehicle (veh), estradiol benzoate (EB), or EB plus progesterone (EBP). Protein levels were similar across groups. Bars = 500 µm.

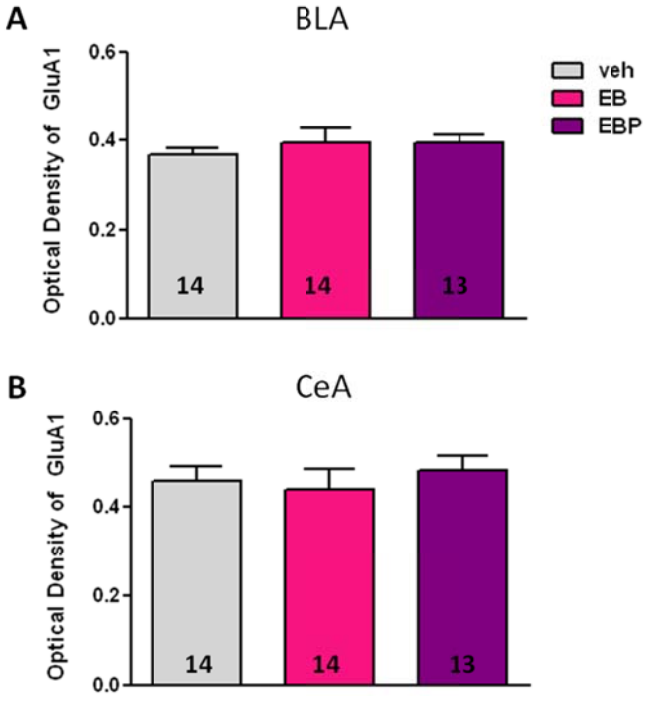

Figure 4.18. Quantification of GluA1 levels.

There were no hormone effects in either brain area observed.

A. Optical density of GluA1 in the BLA.

B. Optical density of GluA1 in the CeA.
Numbers in bars represent sample size.

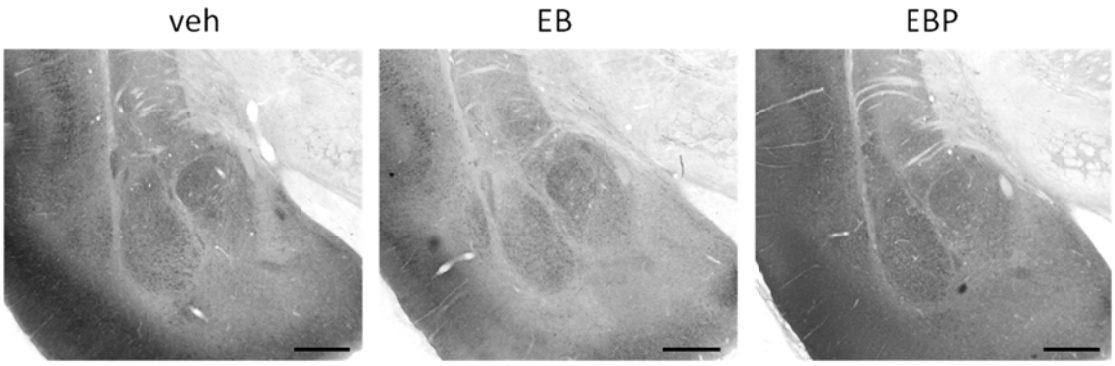

Figure 4.19. Photomicrographs of GluA2 immunoreactivity in the amygdala of rats treated with vehicle (veh), estradiol benzoate (EB), or EB plus progesterone (EBP). Protein levels were reduced in the EB condition. Bars = 500 µm.

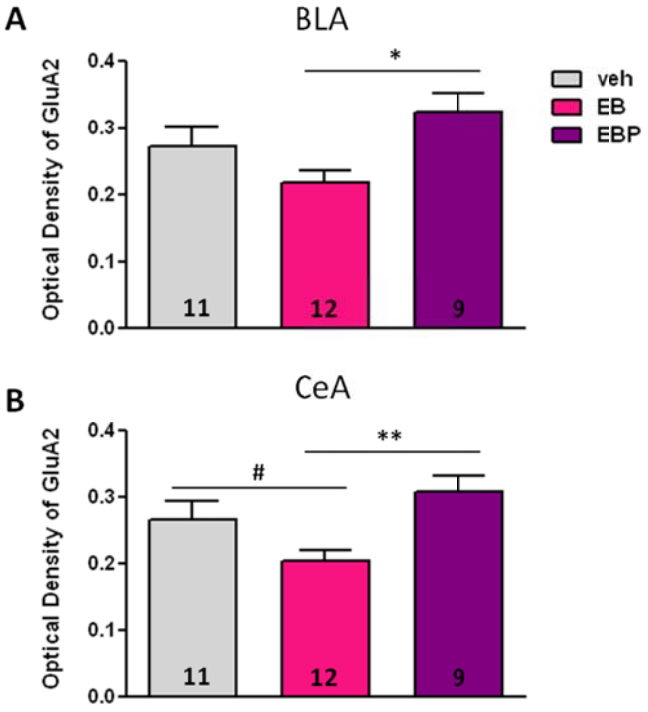

Figure 4.20. Quantification of GluA2 levels.

A. Optical density of GluA1 in the BLA. Estradiol significantly decreased GluA2 in the BLA. *, $p < 0.05$ compared to EBP.

B. Optical density of GluA1 in the CeA. There was a trend for estradiol to decrease GluA2 compared to vehicle treatment, #, $p < 0.10$. Estradiol decreased GluA2 compared to treatment with estradiol plus progesterone. **, $p < 0.01$ compared to EBP.

Numbers in bars represent sample size.

Early hormone effects-cofilin in the VMH

Because phosphorylation of cofilin is necessary for spinogenesis (Kramar et al., 2009 and 2012; Rochefort and Konnerth, 2012; Christensen et al., 2011; Schwarz et al, 2008) but no changes were seen at 76 hours when dendritic spines are observed in the vlVMH, we

hypothesized that p-cofilin levels would be increased at earlier timepoints, either preceding the appearance of spines or concomitant with their earliest emergence, the timing of which is not known. First, to ensure that the circulation of estradiol occurs rapidly after subcutaneous injection, we performed an enzyme-linked immunosorbent assay (ELISA). As expected, acute EB treatment significantly increased plasma estradiol; estradiol-treated rats at 30 minutes and 1 hour had higher estradiol plasma concentrations than vehicle-treated rats at 30 minutes and 1 hour (as the veh 0.5 and veh 1 groups were not different, they were added, as were EB 0.5 and EB 1; t-test, 15.840 ± 2.064 versus 5.407 ± 0.180 pg/ml, $p < 0.004$, Fig. 4.21). Thus, we were able to observe p-cofilin and cofilin immunoreactivity at 30 minutes, one, and four hours after hormone treatment, which is shown in Fig. 4.22 and 4.23, and quantified in Fig. 4.24A-C, 4.25A-C, and 4.26A-C, respectively. For p-cofilin levels in the vlVMH, a two-way ANOVA revealed a significant main effect of time ($F_{(2,36)} = 3.697$; $p < 0.040$) and a treatment x time interaction ($F_{(2,36)} = 8.384$; $p < 0.002$). More specifically, there was a trend for estradiol to increase p-cofilin levels from control levels within 30 minutes (0.338 ± 0.012 versus 0.257 ± 0.032 AU, $p < 0.100$), while at one hour after estradiol administration, p-cofilin decreased to vehicle level of expression, and at four hours, estradiol significantly decreased p-cofilin compared to vehicle (0.180 ± 0.009 versus 0.289 ± 0.025 AU, $p < 0.010$, Fig. 4.24A). For total cofilin in the vlVMH, there was a significant effect of time ($F_{(2,31)} = 4.882$; $p < 0.020$), but no hormone effects and no interaction between the two variables (Fig. 4.24B). When considered as a ratio of phosphorylated to total cofilin level, there were no significant main effects of hormone treatment or time, but there was a significant interaction between the two ($F_{(2,31)} = 3.872$; $p < 0.040$). Particularly, estradiol increased the ratio of p-cofilin to total cofilin at 30 minutes (1.099 ± 0.154 versus 0.659 ± 0.103 AU, $p < 0.050$, Fig. 4.24C).

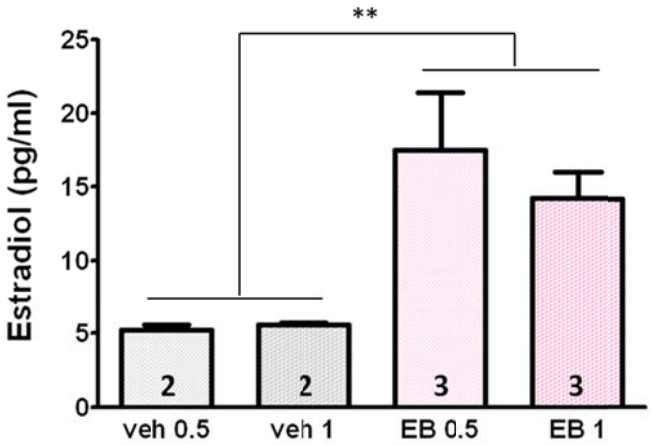

Figure 4.21. Plasma estradiol levels.

Subcutaneous administration of estradiol caused a significant increase in plasma estradiol levels after 30 and 60 minutes compared to vehicle treatment at the same times. **, $p < 0.01$, veh at 30 and 60 minutes, pooled, compared to EB at 30 and 60 minutes, pooled.

Numbers in bars represent sample size.

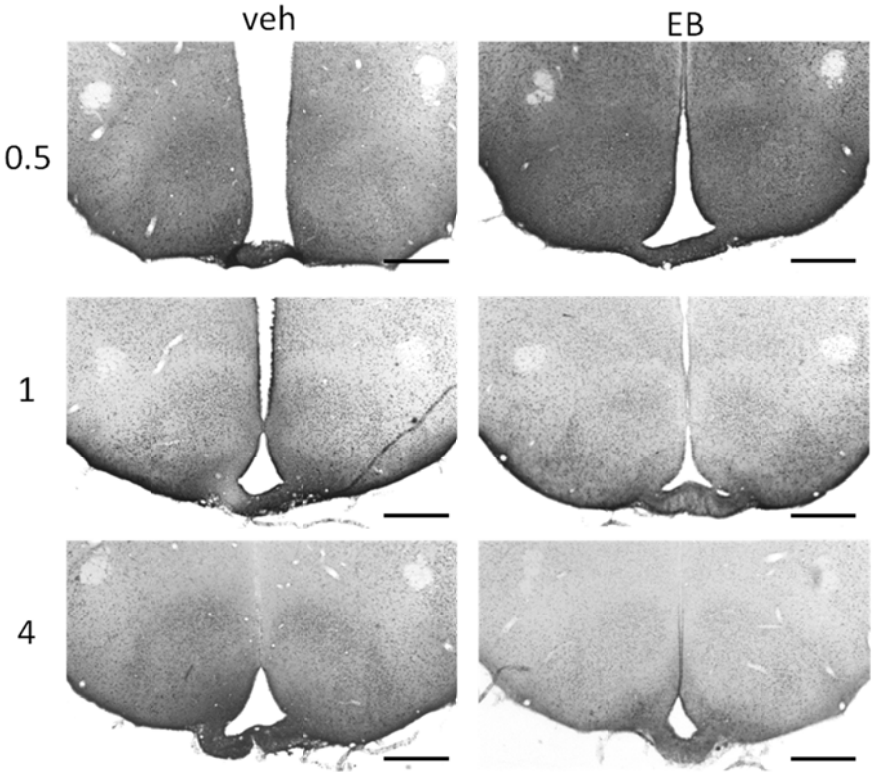

Figure 4.22. Photomicrographs of p-cofilin levels in the VMH.

Photomicrographs of p-cofilin levels in the VMH of rats treated with vehicle (veh) or estradiol benzoate (EB) at 30 minutes, one, or four hours after treatment. Estradiol increased p-cofilin at 30 minutes and decreased it at four hours. Bars = 500 μm.

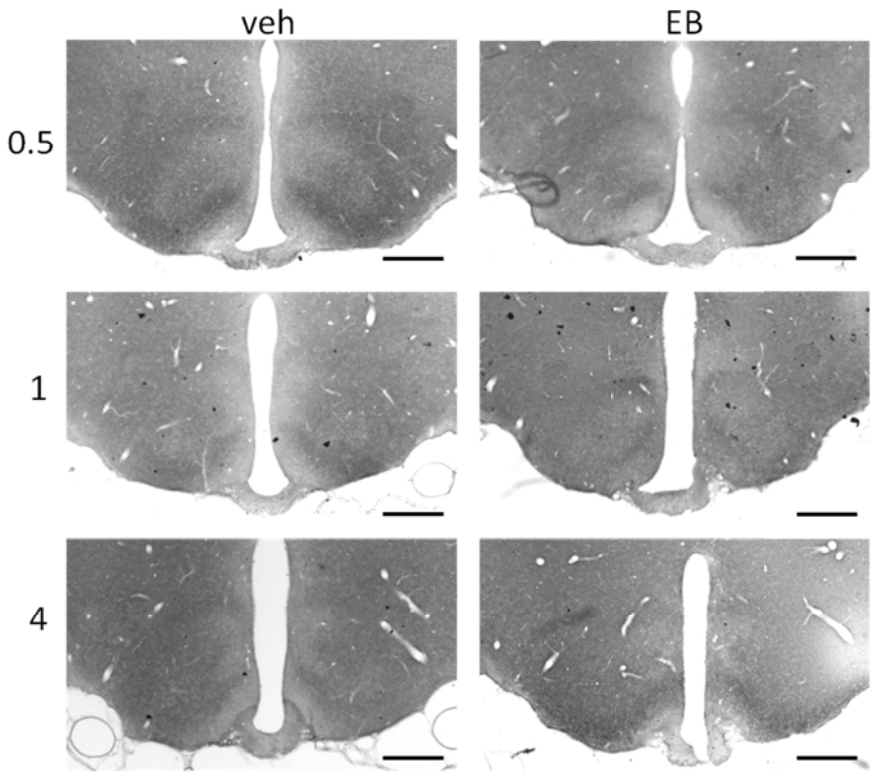

Figure 4.23. Photomicrographs of cofilin immunoreactivity in the VMH.

Photomicrographs of cofilin levels in the VMH of rats treated with vehicle (veh) or estradiol benzoate (EB) at 30 minutes, one, or four hours after treatment. There were no differences across groups at any timepoint. Bars = 500 μm.

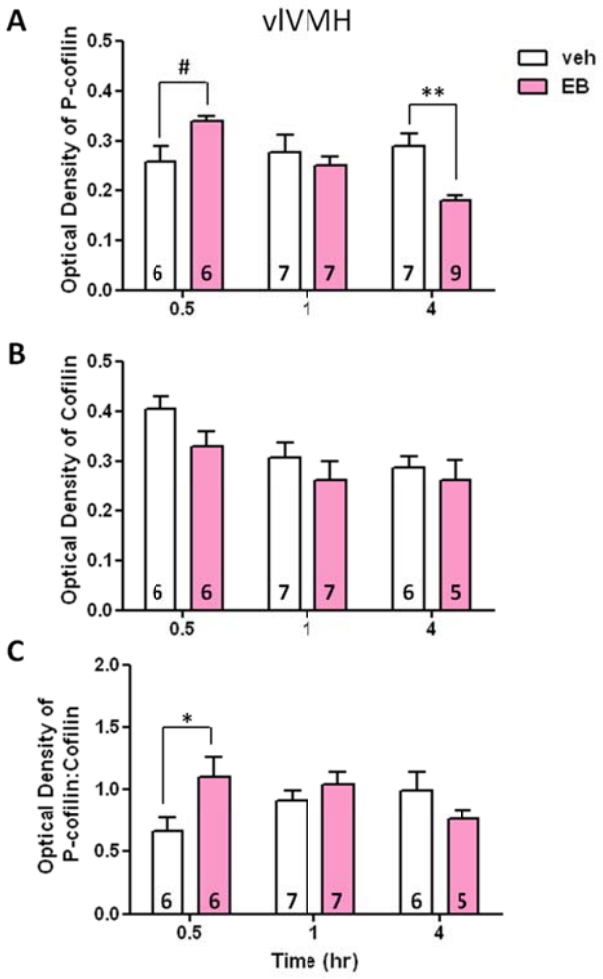

Figure 4.24. Quantification of immunoreactivity in the vlVMH.

A. Optical density of p-cofilin. A two-way ANOVA revealed a significant main effect of time ($F_{(2,36)} = 3.697$; $p < 0.040$) and a treatment x time interaction ($F_{(2,36)} = 8.384$; $p < 0.002$). Particularly, there was a trend for estradiol to increase p-cofilin expression from control levels within 30 minutes (#, $p < 0.10$), while at one hour after estradiol administration, p-cofilin decreased to vehicle level of expression, and at four hours, estradiol significantly decreased p-cofilin compared to vehicle, **, $p < 0.01$.

B. Optical density of cofilin. There was a significant effect of time ($F_{(2,31)} = 4.882$; $p < 0.020$), but no hormone effects at any timepoint.

C. Optical density of the ratio of phosphorylated to total cofilin. A two-way ANOVA revealed a significant interaction between the time and hormone treatment ($F_{(2,31)} = 3.872$; $p < 0.040$). Particularly, estradiol increased the ratio of p-cofilin to total cofilin at 30 minutes, *, $p < 0.050$.

Numbers in bars represent sample size.

For p-cofilin levels in the dmVMH, there was an interaction between time and hormone treatment effects ($F_{(2,36)} = 5.198$; $p < 0.020$), with a significant estradiol-induced decrease at four hours (0.177 ± 0.014 versus 0.273 ± 0.034 AU, $p < 0.050$, Fig. 4.25A). Again, cofilin staining was not altered by hormone treatment at any timepoint (Fig. 4.25B), but there was a significant main effect of time ($F_{(2,31)} = 5.290$; $p < 0.020$). Ratio values in the dmVMH did not reach significance at any timepoint (Fig. 4.25C).

In the LFC, there was a significant main effect on p-cofilin immunoreactivity of time ($F_{(2,37)} = 4.667$; $p < 0.020$) and an interaction between time and hormone treatment ($F_{(2,37)} = 7.144$; $p < 0.003$). Here, estradiol increased p-cofilin immunoreactivity from vehicle levels at 30 minutes (0.305 ± 0.043 versus 0.412 ± 0.031 AU, $p < 0.050$) and decreased it at four hours

(0.229 ± 0.013 versus 0.324 ± 0.013 AU, $p < 0.050$, Fig. 4.26A). Cofilin density in the LFC did not change at 30 minutes, one, or four hours (Fig. 4.26B). There was a trend for time x hormone treatment interaction on the ratio of p-cofilin to cofilin in the LFC ($F_{(2,31)} = 3.170$; $p < 0.060$) and estradiol caused an increase in this ratio at 30 minutes (0.723 ± 0.143 versus 1.311 ± 0.271 AU, $p < 0.050$, Fig. 4.26C).

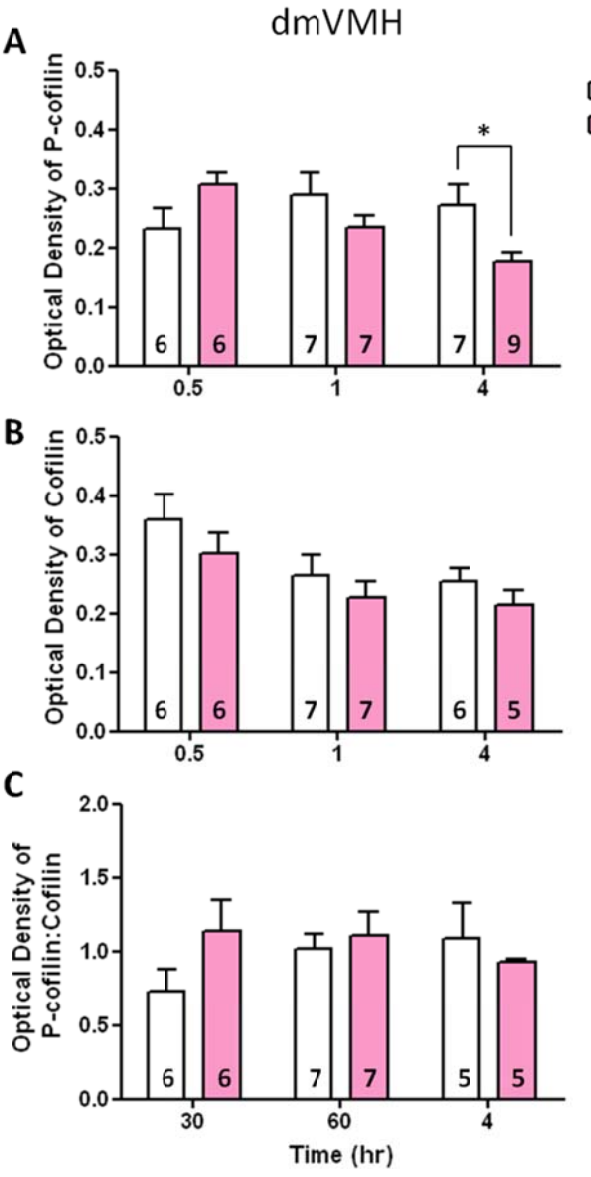

Figure 4.25. Quantification of expression in the dmVMH.

A. Optical density of p-cofilin. There was an interaction between time and hormone treatment effects ($F_{(2,36)} = 5.198$; $p < 0.020$), with a significant estradiol-induced decrease at four hours. *, $p < 0.050$. Ratio values in the dmVMH did not reach significance at any timepoint (Fig. 4.25C).

B. Cofilin staining in the dmVMH was not altered by hormone treatment at any timepoint, but there was a significant main effect of time ($F_{(2,31)} = 5.290$; $p < 0.020$).

C. Optical density of the ratio of phosphorylated to total cofilin. No estradiol-induced changes in the ratio of p-cofilin to total cofilin were observed at 30 minutes in the dmVMH.

Numbers in bars represent sample size.

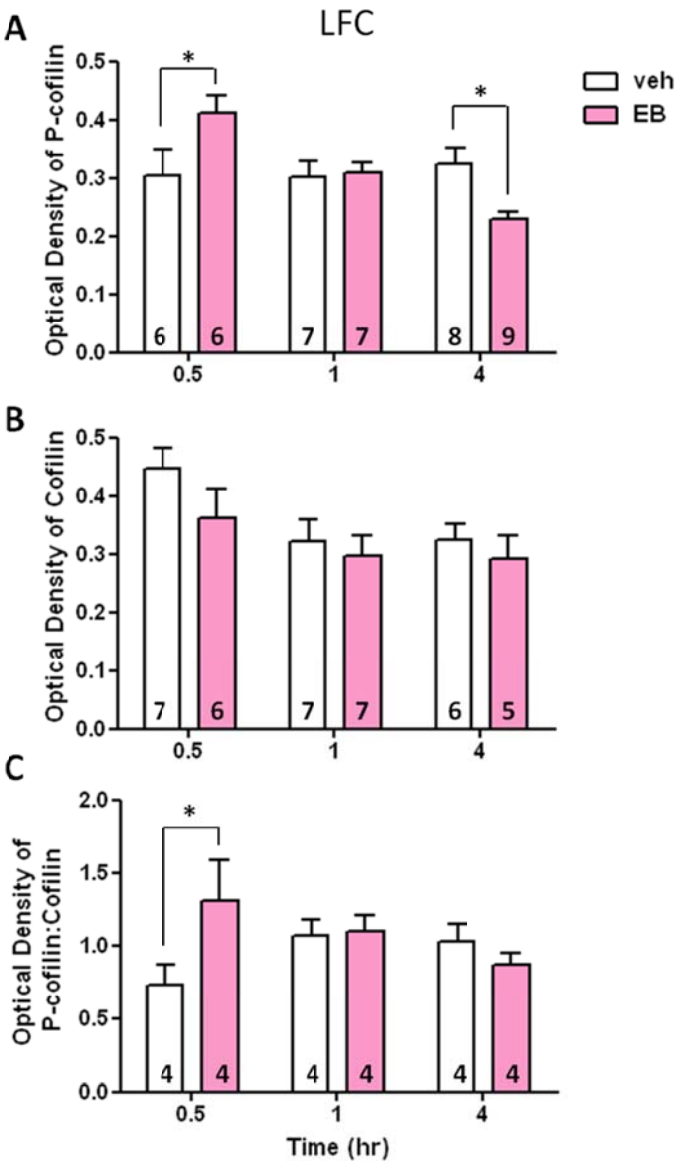

Figure 4.26. Quantification of immunoreactivity in the LFC.

A. Optical density of p-cofilin. There was a significant main effect of time ($F_{(2,37)}$ = 4.667; p < 0.020) and an interaction between time and hormone treatment ($F_{(2,37)}$ = 7.144; p < 0.003). In the LFC, estradiol increased p-cofilin expression from vehicle levels at 30 minutes, *, p < 0.050) and decreased it at four hours, *, p < 0.050, Fig. 4.26A).

B. Optical density of cofilin. Expression did not change at timepoint.

C. Optical density of the ratio of phosphorylated to total cofilin. There was a trend for a main effect of time x hormone treatment on the ratio of p-cofilin to cofilin in the LFC ($F_{(2,31)}$ = 3.170; p < 0.060) and estradiol caused an increase in this ratio at 30 minutes, *, p < 0.05.

Numbers in bars represent sample size.

Early hormone effects-cofilin in the amygdala

P-cofilin and cofilin immunoreactivity in the amygdala is shown in Fig. 4.27A-F and 4.28A-F, respectively. Quantification of the optical density of p-cofilin in the basolateral nucleus of the amygdala (BLA) reveals a significant hormone effect ($F_{(1,38)}$ = 6.889; p < 0.020) and a trend for an interaction between hormone treatment and time ($F_{(2,38)}$ = 3.097; p < 0.060). Specifically, estradiol significantly increases levels after one hour (0.067 ± 0.008 versus 0.117 ± 0.011 AU, p < 0.010, Fig. 4.29A). As in other brain regions, there was a main effect of time on cofilin

immunoreactivity in the BLA ($F_{(2,30)}$ = 6.847; p < 0.004) but estradiol treatment did not affect cofilin immunoreactivity at any timepoint (Fig. 4.29B). A two-way ANOVA of the ratio values in the BLA revealed significant main effects of hormone treatment ($F_{(1,30)}$ = 10.66; p < 0.003) and time ($F_{(2,30)}$ = 5.058; p < 0.020), as well as an interaction between them ($F_{(2,30)}$ = 4.339; p < 0.030). In particular, estradiol induced a robust increase in the ratio of p-cofilin to cofilin at one hour (0.272 ± 0.039 versus 0.580 ± 0.134 AU, p < 0.001, Fig. 4.29C). In the central nucleus of the amygdala (CeA), treatment group had a significant effect on p-cofilin ($F_{(2,37)}$ = 6.878; p < 0.020) and there was a trend for an interaction between hormone treatment group and time ($F_{(2,37)}$ = 2.474; p < 0.100). At one hour, estradiol caused a significant increase in p-cofilin density (0.075 ± 0.009 versus 0.123 ± 0.016 AU, p < 0.050, Fig. 4.30A). Cofilin levels exhibited a significant main effect of time ($F_{(2,28)}$ = 8.140; p < 0.002) but values were similar between veh and EB groups at all timepoints (Fig. 4.30B). In the CeA there was a significant effect of hormone treatment ($F_{(1,28)}$ = 12.54; p < 0.002) and time ($F_{(2,28)}$ = 5.436; p < 0.020) on p-cofilin:cofilin. Specifically, the ratio was increased at one hour after estradiol administration (0.292 ± 0.034 versus 0.785 ± 0.236 AU, p < 0.010, Fig. 4.30C).

Early hormone effects-AMPAR subunits in the VMH

As AMPAR subunits GluA1 and 2 were moderated by ovarian hormones at later timepoints when spines have been shown to be altered, we investigated whether these changes occurred earlier than 76 hours after administration and whether they preceded, followed, or were concomitant with changes in cofilin. However, due to antibody availability, GluA2 levels could not be assessed. GluA1 immunoreactivity in the vlVMH, dmVMH, and LFC at 30 minutes, one, and four hours in veh and EB conditions is depicted in Fig. 4.31. Analysis of the vlVMH revealed a significant hormone treatment effect ($F_{(1,31)}$ = 4.736; p < 0.040) and an interaction between hormone treatment and time ($F_{(2,31)}$ = 3.844; p < 0.040). Bonferroni post hoc test uncovered that estradiol caused a decrease in GluA1 after four hours (0.483 ± 0.037 versus 0.354 ± 0.023 AU, p < 0.010, Fig. 4.32A). There was a similar, but less robust effect in the dmVMH, with a trend

towards a main hormone effect ($F_{(1,31)}$ = 3.636; p < 0.070). At four hours estradiol decreased GluA1 (0.462 ± 0.033 versus 0.349 ± 0.019 AU, p < 0.050, Fig. 4.32B). There were no differences in GluA1 immunoreactivity at any timepoint in the LFC (Fig. 4.32C).

Early hormone effects-AMPAR subunits in the amygdala

GluA1 staining in the amygdala is represented in Fig. 4.33. In the BLA, a significant time ($F_{(2,31)}$ = 5.010; p < 0.020) and treatment ($F_{(1,31)}$ = 15.18; p < 0.0006) effect were present. Specifically, there was an estradiol-induced decrease in GluA1 at 30 minutes (0.492 ± 0.019 versus 0.379 ± 0.035 AU, p < 0.050) and at four hours (0.403 ± 0.024 versus 0.272 ± 0.011 AU, p < 0.011, Fig. 4.34A). A two-way ANOVA of GluA1 density in the CeA yielded significant time ($F_{(2,31)}$ = 4.801; p < 0.020) and treatment ($F_{(1,31)}$ = 18.68; p < 0.0002) main effects. A Bonferroni post-hoc test revealed that GluA1 was decreased in response to estradiol at 30 minutes post-treatment (0.447 ± 0.027 versus 0.303 ± 0.017 AU, p < 0.050, Fig. 4.34B).

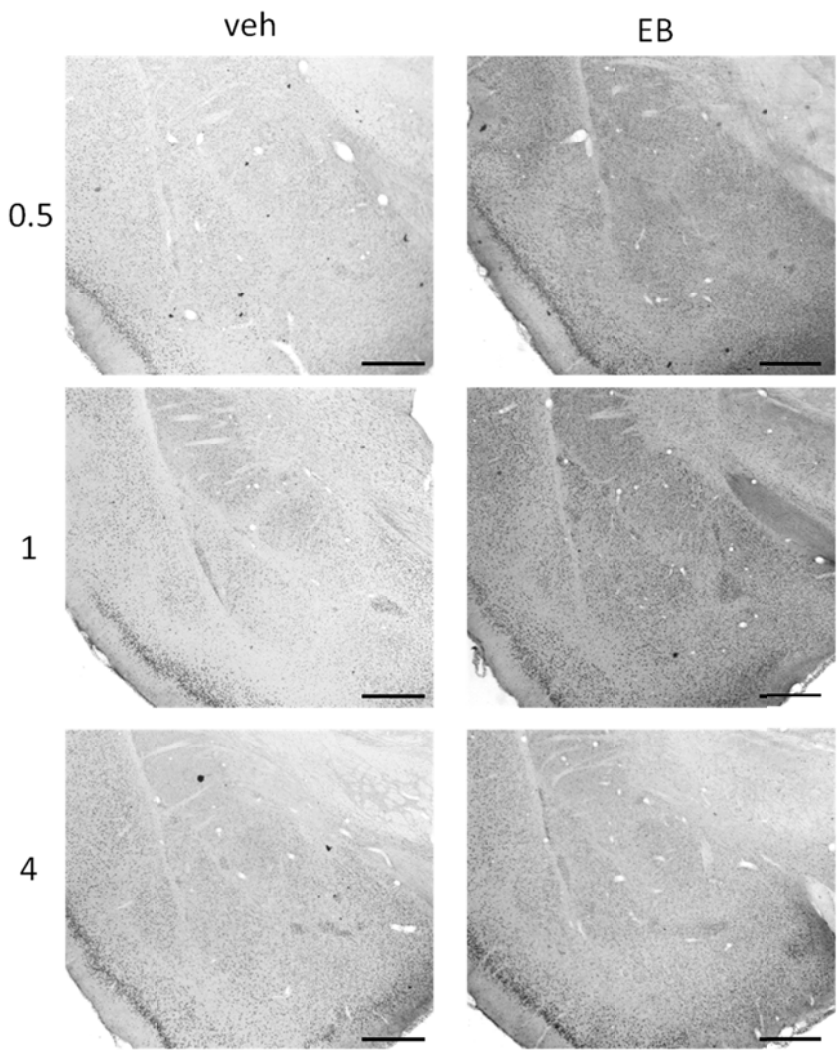

Figure 4.27. Photomicrographs of p-cofilin immunoreactivity in the amygdala.

Photomicrographs of p-cofilin levels in the amygdala of rats treated with vehicle (veh) or estradiol benzoate (EB) at 30 minutes, one, or four hours after treatment. Estradiol increased p-cofilin one hour after treatment.
Bars = 500 µm.

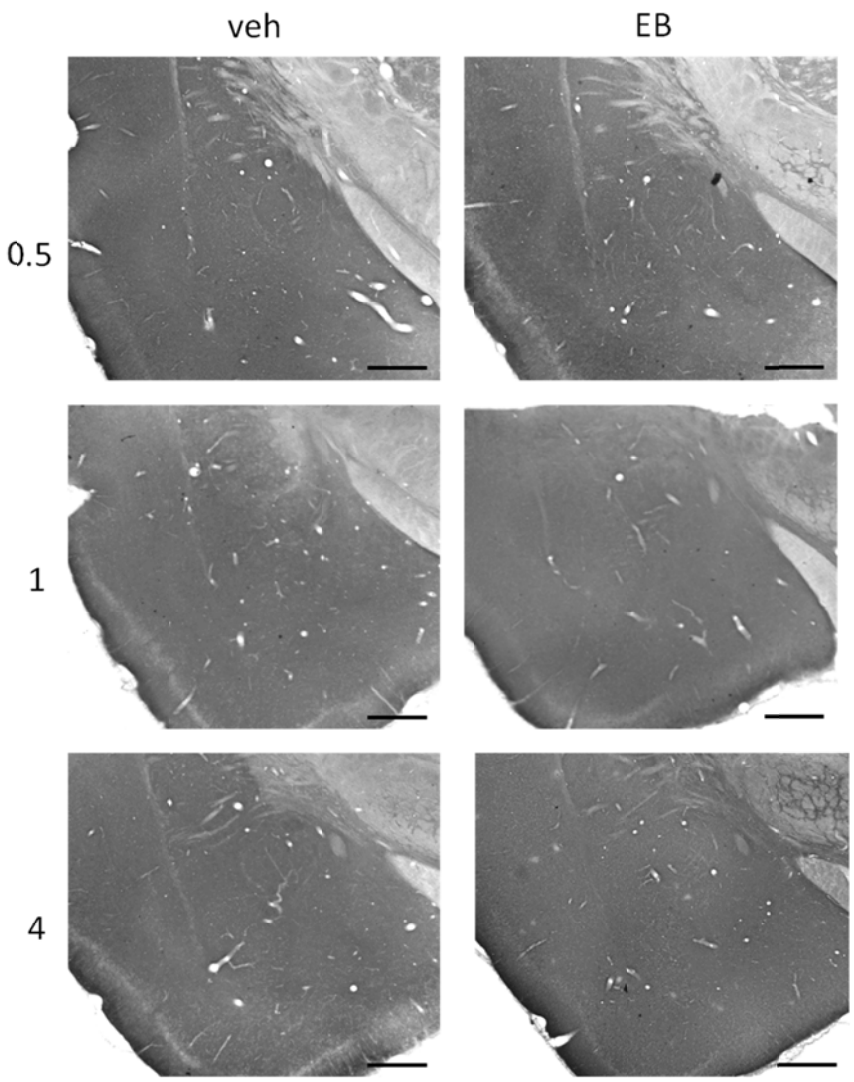

Figure 4.28. Photomicrographs of cofilin immunoreactivity in the amygdala.
Photomicrographs of cofilin immunoreactivity in the amygdala of rats treated with vehicle (veh) or estradiol benzoate (EB) at 30 minutes, one, or four hours after treatment. Estradiol did not change staining density at any timepoint.
Bars = 500 µm.

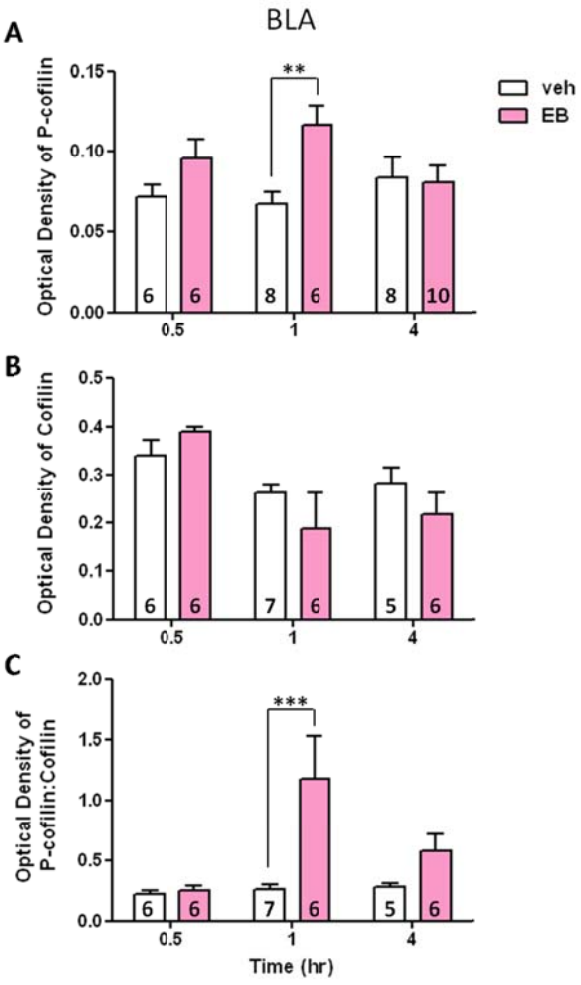

Figure 4.29. Quantification of immunoreactivity in the BLA.

A. Optical density of p-cofilin. There was a significant hormone effect ($F_{(1,38)}$ = 6.889; p < 0.020) and a trend for an interaction between hormone treatment and time ($F_{(2,38)}$ = 3.097; p < 0.060). Specifically, estradiol significantly increased p-cofilin after one hour. **, p < 0.01.

B. Optical density of cofilin. Cofilin staining in the dmVMH was not altered by hormone treatment at any timepoint, but there was a significant main effect of time ($F_{(2,30)}$ = 6.847; p < 0.004).

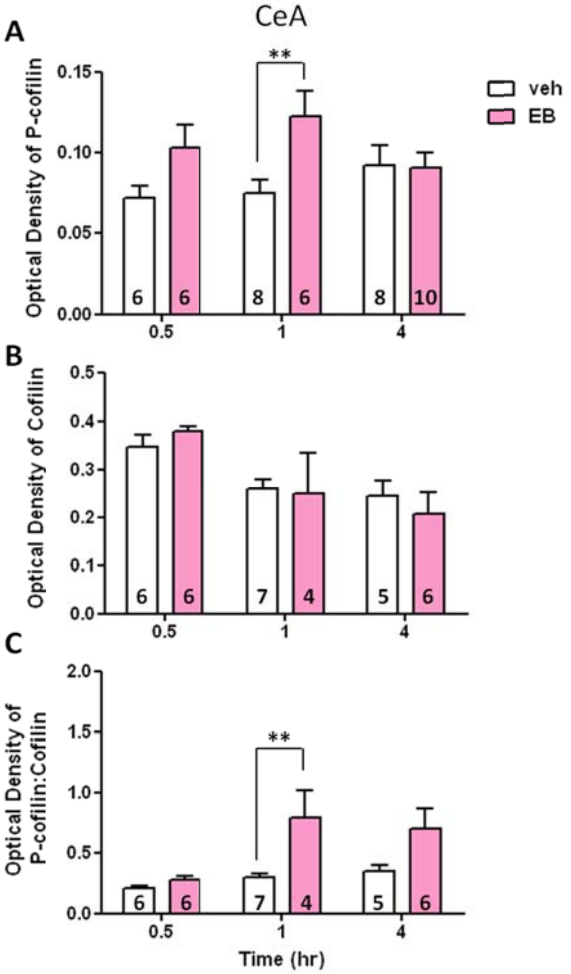

Figure 4.30. Quantification of immunoreactivity in the CeA.

A. Optical density of p-cofilin. Treatment group had a significant effect on p-cofilin ($F_{(2,37)}$ = 6.878; p < 0.020) and there was a trend for an interaction between hormone treatment and time ($F_{(2,37)}$ = 2.474; p < 0.100). Post hoc tests revealed that at one hour, estradiol caused a significant increase in p-cofilin density, **, p < 0.01.

B. Optical density of cofilin expression. Cofilin expression exhibited a significant main effect of time ($F_{(2,28)}$ = 8.140; p < 0.002) but values were similar between veh and EB groups at all timepoints.

C. Optical density of the ratio of phosphorylated to total cofilin. A two-way ANOVA revealed significant main effects of hormone treatment ($F_{(1,30)} = 10.66$; $p < 0.003$) and time ($F_{(2,30)} = 5.058$; $p < 0.020$), as well as an interaction between them ($F_{(2,30)} = 4.339$; $p < 0.030$). In particular, estradiol induced a robust increase in the ratio of p-cofilin to cofilin at one hour, ***, $p < 0.001$.

Numbers in bars represent sample size.

C. Optical density of the ratio of phosphorylated to total cofilin. A two-way ANOVA revealed a significant effect of hormone treatment ($F_{(1,28)} = 12.54$; $p < 0.002$) and time ($F_{(2,28)} = 5.436$; $p < 0.020$). Specifically, the ratio was increased at one hour after estradiol administration. **, $p < 0.010$.

Numbers in bars represent sample size.

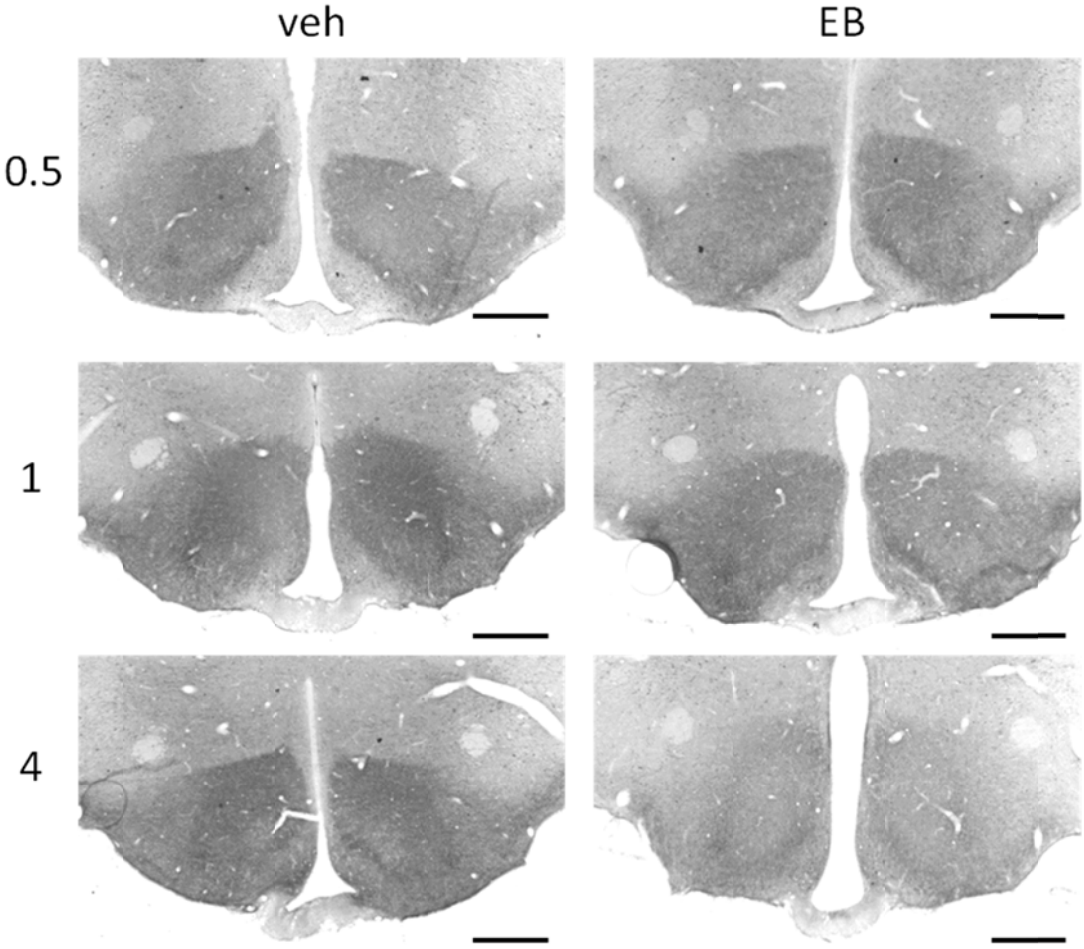

Figure 4.31. Photomicrographs of GluA1 immunoreactivity in the VMH of rats treated with vehicle (veh) or estradiol benzoate (EB) at 30 minutes, one, or four hours after treatment. Estradiol decreased GluA1 four hours after treatment.
Bars = 500 µm.

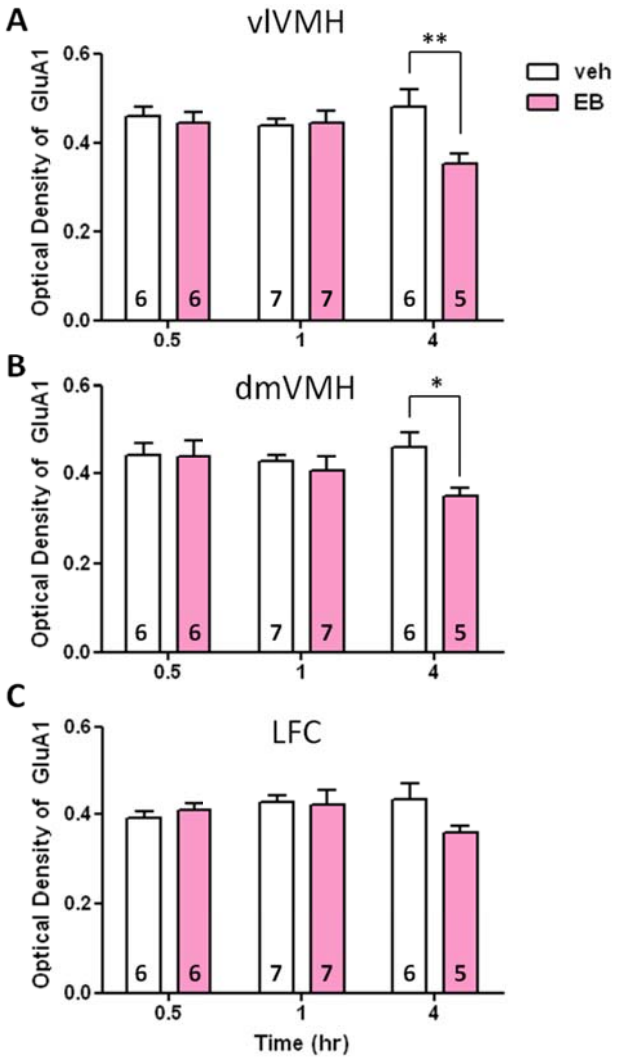

Figure 4.32. Quantification of GluA1 immunoreactivity in the hypothalamus.
A. Optical density of GluA1 in the vlVMH. A two-way ANOVA revealed a significant main effect of hormone treatment ($F_{(1,31)} = 4.736$; $p < 0.040$) and an interaction between hormone treatment and time ($F_{(2,31)} = 3.844$; $p < 0.040$). Bonferroni post hoc test uncovered that estradiol caused a decrease in GluA1 after four hours. **, $p < 0.01$.

B. Optical density in the dmVMH. There was a trend towards a main hormone effect ($F_{(1,31)} = 3.636$; $p < 0.070$). At four hours estradiol decreased GluA1, *, $p < 0.050$.

C. Optical density in the LFC. There were no differences in GluA1 levels at any timepoint in the LFC.

Numbers in bars represent sample size.

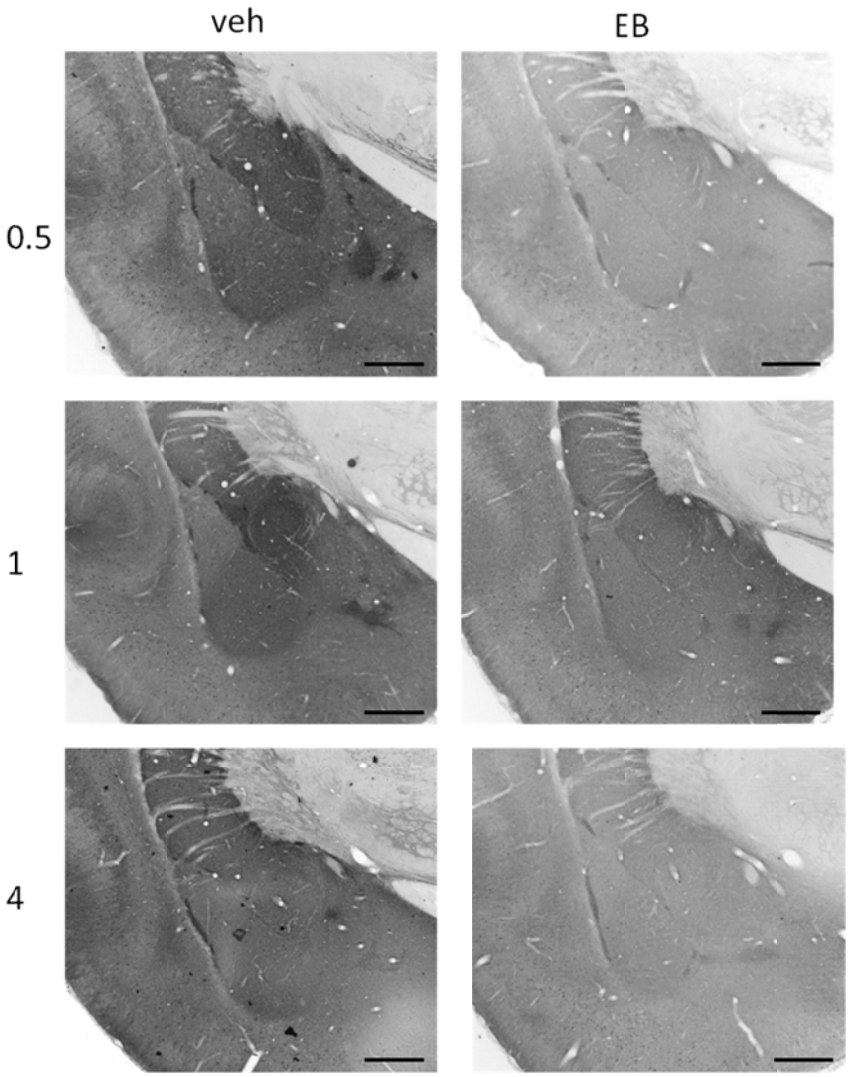

Figure 4.33. Photomicrographs of GluA1 immunoreactivity in the amygdala of rats treated with vehicle (veh) or estradiol benzoate (EB) at 30 minutes, one, or four hours after treatment. Estradiol decreased GluA1 30 minutes and four hours after treatment. Bars = 500 μm.

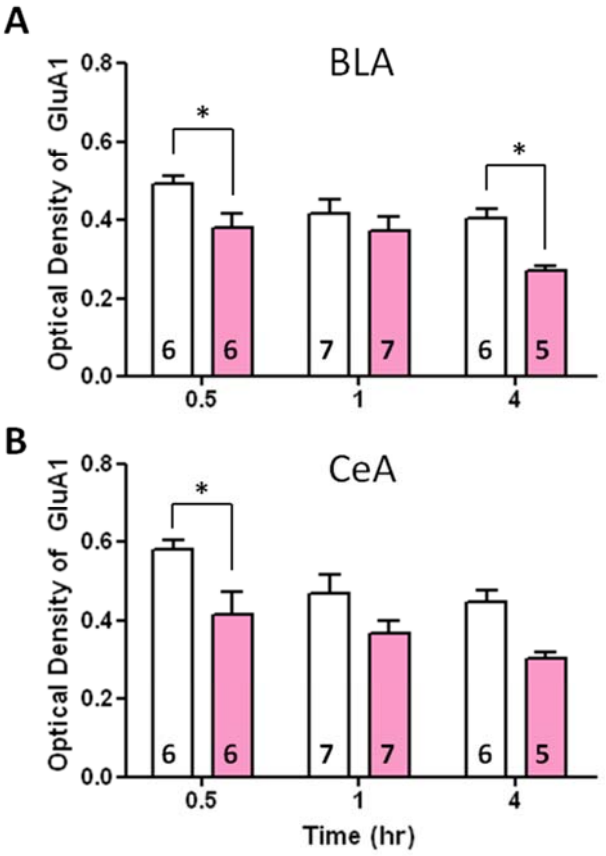

Figure 4.34. Quantification of GluA1 immunoreactivity in the amygdala.

A. Optical density of GluA1 in the BLA. Significant time ($F_{(2,31)}$ = 5.010; p < 0.020) and treatment ($F_{(1,31)}$ = 15.18; p < 0.0006) effects were present. Specifically, there was an estradiol-induced decrease in GluA1 at 30 minutes and at four hours. *, p < 0.05.

B. Optical density of GluA1 in the CeA. A two-way ANOVA of GluA1 density yielded significant time ($F_{(2,31)}$ = 4.801; p < 0.020) and treatment ($F_{(1,31)}$ = 18.68; p < 0.0002) main effects. A Bonferroni post-hoc test revealed that GluA1 was decreased in response to estradiol at 30 minutes post-treatment. *, p < 0.05.

Discussion

Estradiol-induced changes in spine density are well documented in the vlVMH (Nishizuka and Pfaff, 1989; Frankfurt et al., 1990; Frankfurt and McEwen, 1991; Segarra and McEwen, 1991; Calizo and Flanagan-Cato, 2000; Calizo and Flanagan-Cato, 2002) and these structural alterations are likely important for the execution of lordosis. However, the timing and mechanisms involved are not known. This study investigated immunohistochemical expression of the actin depolymerizing protein cofilin, its phosphorylated form, which promotes actin assembly, and the plasticity-associated AMPAR subunits GluA1 and 2 over multiple timepoints after ovarian hormone administration, as these factors have all been implicated in dendritic spine formation (Rochefort and Konnerth, 2012; Kramar et al., 2009; Kramar et al., 2012; Christensen et al., 2011; Passafaro et al., 2003; McKinney et al., 1999; Richards et al., 2005; Medvedev et al., 2008; Schwarz et al., 2008).

The specific goal of this experiment was to test the hypotheses that 1) estradiol would rapidly increase the immunoreactivity levels of phosphorylated cofilin (p-cofilin) and the ionotropic glutamate AMPA receptor subunit GluA1 and 2) estradiol and progesterone modulate GLuA2 at later timepoints when females are sexually receptive and hormone-induced spines are present on vlVMH dendrites. I found that estradiol increases phosphorylated cofilin relative to total cofilin levels (p-cofilin:cofilin) in the vlVMH and LFC in 30 minutes. At one hour, estradiol increases p-cofilin:cofilin in the BLA and CeA. By four hours after estradiol treatment, GluA1 levels decrease in the vlVMH, dmVMH, and LFC. At 76 hours, estradiol decreases GluA2 in the vlVMH, BLA, and CeA while the addition of progesterone increases GluA1 in the vlVMH and LFC.

Cofilin and p-cofilin

While in the cases of both p-cofilin and cofilin staining, the boundaries of the VMH are clear, p-cofilin immunoreactivity is punctate while cofilin immunoreactivity appears more diffuse (Fig. 4.1B and Fig. 4.3A-B, respectively). A closer examination of p-cofilin will be necessary to determine whether it is expressed in dendritic spines specifically. Although p-cofilin was punctate

in nature, it did not appear to be expressed in nuclei, and therefore, number of particles were not counted because they would not necessarily reflect the number of cells expressing p-cofilin. Moreover, optical density is a standard measure that can be applied to both p-cofilin and cofilin levels in order to yield a ratio. The ratio of phosphorylated to total cofilin is arguably the most informative quantification because while cofilin levels seem to be somewhat dynamic—for example, they are not modulated by estradiol but tend to decrease over time, even in the vehicle condition (Fig. 4.24B)—the ratio reflects the percentage of proteins that are phosphorylated and thus would allow actin polymerization, which is an essential step in spinogenesis. Therefore, an increase in p-cofilin:cofilin would be expected during spine formation.

Female sexual behavior is at its peak when exogenous hormones are administered in a regimen that mimics the proestrus phase of the estrous cycle. Specifically, females exhibit lordosis four hours after a progesterone treatment, which is given 72 hours after the first of two doses of estradiol spaced 24 hours apart. Females' resulting reproductive window lasts only a few hours (Boling and Blandau, 1939). At this timepoint, 76 hours after an initial estradiol administration, dendritic spine density is increased in the vlVMH, the nucleus most strongly associated with reproductive behavior in numerous species (Frankfurt et al., 1990; Madeira et al., 2005; Calizo and Flanagan-Cato, 2000; Griffin and Flanagan-Cato, 2011). Because phosphorylation of cofilin is necessary for estradiol-dependent spine formation according to several studies involving the hippocampus and arcuate nucleus of the hypothalamus (Kramar et al., 2009 and 2012; Rochefort and Konnerth, 2012; Christensen et al., 2011; Schwarz et al., 2008), we hypothesized that p-cofilin and p-cofilin:cofilin would be increased at this time. Interestingly, we saw no change in p-cofilin, cofilin, or the ratio of the former to the latter at 76 hours after estradiol administration or with the addition of progesterone.

Because increases in phosphorylation of cofilin were not concurrent with the timing of observed spine changes, we hypothesized that such an increase may take place earlier. First, we confirmed the efficacy of rapid estradiol circulation after injection via ELISA of plasma estrogen levels (Fig. 4.21), then we analyzed optical density at 30 minutes, one hour, and four

hours. We chose these timepoints based on a previous study in which estradiol induced an increase in p-cofilin in one hour and an increase in spines in four hours in the adjacent arcuate nucleus of the hypothalamus, which was attributed to probable rapid membrane estrogen receptor action (Christensen et al., 2011). These are the earliest timepoints surveyed in the hypothalamus and we wanted to include an even earlier timepoint.

In the present study in the vlVMH we found a trend for increased p-cofilin and a significant increase in p-cofilin:cofilin at 30 minutes after estradiol administration. In both cases, by one hour levels had returned to control levels, and by four hours p-cofilin was significantly decreased compared to vehicle treatment. However, this effect was lost when expressed as a ratio of total protein (Fig. 4.24A-C). Increasing sample size may help clarify what is happening at four hours. If there is a decrease in phosphorylation at four hours, it could be a result of feedback or reflect a depletion of energy stores for phosphorylation events after increased activity. Additionally, phosphorylation may occur rapidly and begin to decline by 30 minutes, accounting for the modest effect in p-cofilin observed here. The effects of progesterone on cofilin staining were not examined here, so this will be an interesting focus for future experiments. While it will also be important to examine p-cofilin before 30 minutes to help further clarify the timecourse of changes in protein levels, the ratio of phosphorylated to unphosphorylated cofilin is significantly increased 30 minutes after estradiol treatment. Finally, while it cannot be discounted that further changes occur between the times we observed here, it seems likely that estradiol-induced phosphorylation of cofilin in the vlVMH is a rapid and transient effect that likely contributes to the later emergence of dendritic spines.

The discrepancy between when p-cofilin changes occur and when spines are observed may indicate that cofilin phosphorylation occurs at the beginning of the spinogenesis cascade, and while phosphorylation events occur rapidly, manifestation of structural changes, in the form of new spines, requires more time. This may be evident in an increase in newly formed filopodeal spines at 30 minutes. Phosphorylation levels may then decline as spines stabilize or mature by 76 hours, assuming the mushroom and stubby conformations. Another explanation for the lack of

coordination of p-cofilin and spine changes in the vlVMH may have to do with the spatial specificity of morphology at this time. As discussed previously, dendritic spine density is increased on short primary dendrites, but decreased on long primary dendrites that project to the periaqueductal gray (PAG). Therefore, phosphorylation may be increasing on some dendrites and decreasing on others, diminishing an overall effect of increased p-cofilin. Confocal or electron microscopy may be useful tools to explore this possibility in the future. While further study of cofilin phosphorylation in the vlVMH is warranted, these data provide an indication that this step is likely an important catalyst to spinogenesis, occurs more rapidly than previously thought, precedes structural changes, and is mediated by estradiol.

The LFC, like the vlVMH, failed to exhibit changes in p-cofilin or p-cofilin:cofilin at 76 hours after estradiol administration or after the addition of progesterone. At earlier timepoints, the LFC displayed similar changes as those observed in the vlVMH, however, the increase in p-cofilin at 30 minutes reached significance and remained significant as a ratio of total cofilin. There was also a decrease in p-cofilin four hours after estradiol administration, but this was not significant when expressed as a ratio, indicating that an increase in sample size would be beneficial to clarify specific changes at this time. The LFC contains few to no cell bodies, and mostly consists of fibers that project to the pituitary and also make synaptic contact with and provide input to the vlVMH dendrites. Consequently, any effects observed in this area would likely reflect structural changes occurring in dendrites extending from the vlVMH. Therefore, the increase in p-cofilin in the LFC may seem surprising because spine density decreased on long primary dendrites that send axonal projections to the periaqueductal gray (PAG) in response to estradiol (Calizo and Flanagan-Cato, 2002). Additionally, as the estradiol-induced increase in spine density was observed on short primary dendrites (Calizo and Flanagan-Cato, 2000), which do not extend into the LFC, a decrease in p-cofilin might have been expected. However, there are many long primary dendrites that do not project to the PAG and our results may indicate an increase in spines on long primary dendrites of another as yet uncharacterized subset, or a combination of various dendrites of a heterogeneous group of vlVMH cell types. This seems likely, as cell

populations are poorly defined in the vlVMH. These uncertainties necessitate a more thorough investigation of vlVMH cell type and spine changes on dendrites that extend into the LFC. However, this is one of the few studies to examine important ovarian hormone-induced molecular changes in this fiber plexus, which exhibits estradiol-mediated synaptic reorganization (Griffin et al., 2010), deeming it an important region in the lordosis circuit.

In the dmVMH, no changes in p-cofilin or p-cofilin:cofilin were observed 76 hours after estradiol administration or in the progesterone condition, although the sample sizes of ratio quantifications were fairly small. Somewhat surprisingly, estradiol significantly decreased p-cofilin at four hours. This was not expected because spine changes have been observed exclusively in the vlVMH, not the dmVMH (Madeira et al., 2001; Calizo and Flanagan-Cato, 2000). It is possible that a previously undetermined cell population does in fact undergo estradiol-induced spinogenesis. Because Golgi and cell-filling techniques tend to target random cell types, a subset of dendrites that exhibit a spine density increase in response to estradiol may not have been detected. However, it is important to note that estradiol did not have a significant effect on the ratio of phosphorylated to total cofilin at any timepoint. Therefore, it is likely that no estradiol-induced spinogenesis occurs in this brain area, as expected. The changes in p-cofilin may be a product of variability in the dmVMH. Replication of these results may help reinforce instability in this region, but the current data suggest that there is no clear evidence of estradiol responsivity or spine changes in the dmVMH.

To ensure that changes in protein levels were specific and not global, we included an analysis of the basolateral and central amygdaloid nuclei. One hour after estradiol administration there was a significant increase in p-cofilin in both the BLA and CeA. The ratio of phosphorylated to total cofilin was significantly increased in response to estradiol treatment at one hour in both areas as well. These results are interesting because while estradiol-induced spinogenesis has been reported in the medial nucleus of the amygdala (de Castilhos et al., 2008), ovarian hormone effects on spines have not been investigated in the BLA and CeA. These data suggest that estradiol may increase dendritic spine density in these areas as well. However, chronic restraint

stress results in increased dendritic spines (Mitra et al., 2005), while systemic and intra-amygdalar estradiol produce anxiolytic effects (Frye and Walf, 2004; Mora et al., 1996). Therefore, one might expect estradiol to decrease p-cofilin:cofilin in the BLA and CeA. Thus, additional research will be necessary to determine whether spines are changing in the BLA and CeA in response to estradiol and the timing involved. In any event, our results show that the amount of phosphorylated cofilin relative to total cofilin seems to begin to increase mildly at 30 minutes, exhibits a robust spike by one hour after estradiol treatment, and begins to return to baseline levels by four hours in both the BLA and CeA. Interestingly, changes in these brain regions occur after those observed in the hypothalamus.

While estradiol did not significantly affect total cofilin levels at any timepoint in any brain region, there was a pattern of immunoreactivity in all brain areas analyzed for cofilin to modestly decrease from the 30 minute point to the one and four hour timepoints. This may indicate a circadian effect, but that seems unlikely as similar results were obtained when injections and sacrifices were performed at somewhat variable times. While all procedures were performed within several hours of one another, there was no statistical difference between levels from rats injected in the light versus dark cycle. Similarly, the time effect may be due to food intake, as hormone injections were administered shortly before or after rats' active phase, when food intake also increases. While such an effect may be expected in the dmVMH, which has been associated with feeding behavior, it may not be expected in the vlVMH, and seems even less likely to be reflected in the amygdala. Another possibility is that cofilin is increased shortly after injection in response to stress, however, subcutaneous injections are well tolerated by the rats and effects of stress would not be expected to be evident in the hypothalamus. Further investigation may be helpful to dismiss this possibility.

AMPAR subunits

It is interesting that GluA1 levels in the vlVMH decrease at four hours, and then increase at 76 hours after the addition of progesterone. It is important to uncover what dynamics are

taking place in between those timepoints. GluA2 is decreased 76 hours after estradiol treatment, but is restored to baseline levels after the addition of progesterone. Unfortunately, we were not able to examine GluA2 immunoreactivity at earlier timepoint because of antibody unavailability, but future studies should address this absence.

Hormone-regulated changes in GluA1 and GluA2 at 76 hours or in the estradiol plus progesterone condition, when spines are increased, are suggestive that these changes occur downstream of cofilin phosphorylation events in the vlVMH and LFC. It is plausible that the changes we observed reflected receptor subunits being inserted into or expressed on new, estradiol-induced spines. Previous work has indicated a likelihood for AMPAR insertion following spine stabilization by correlation of AMPAR number with number of mushroom-shaped spines (Matsuzaki et al., 2001). Therefore, it is possible that GluA1 and GlA2 responses to estradiol manifest after initial spine formation has already taken place.

While we can't completely rule out the possibility that existing AMPAR are necessary for spinogenesis, our recent pilot study utilizing antagonists indicates that AMPAR are not necessary for the rapid estradiol-induced increase in p-cofilin. We administered the AMPAR antagonist CNQX or the N-methyl-D-aspartate receptor (NMDAR) antagonist AP-5 intracerebroventricularly (icv) 15 minutes prior to estradiol treatment. When p-cofilin, cofilin, ratio, and GluA1 optical density levels were surveyed 30 minutes later, no changes were observed. In all brain regions examined (vlVMH, dmVMH, LFC, BLA, and CeA), protein immunoreactivity levels were the same in the estradiol alone groups as the antagonist plus estradiol groups (Fig. A.1-A.2). Therefore, while this is suggestive that AMPAR and NMDAR are not necessary for estradiol-mediated changes in the cofilin pathway, these drugs were given icv at concentrations that facilitated lordosis behavior when administered to the vlVMH (Georgescu and Pfaus, 2006). Thus, replication of these results utilizing a parenchymal injection in order to concentrate the effects may be informative. Additionally, the pharmacological intervention here may have been effective if performed at different timing, more or less than 15 minutes before estradiol administration. Finally, confocal microscopy would be a useful tool to determine whether the AMPAR are

localized to spines. In conclusion, GluA1 and GluA2 are both altered at the time of increase in spine density and receptivity, and we hypothesize that AMPAR are inserted into newly formed spines in the vlVMH, which are a result of early cofilin phosphorylation.

Although results from the present immunohistochemical timing study suggest that changes in AMPAR are activated after estradiol catalyzes cofilin phosphorylation to induce spinogenesis, several groups have suggested that AMPAR are necessary for, and thus precede, spine formation. Overexpression of GluA2 causes an increase in spine number, length, and width in hippocampal cells and induces spines on previously nonspiny GABA interneurons (Passafaro et al., 2003). Likewise, AMPAR antagonists and GluA2-siRNA decrease spine formation in hippocampal slice cultures (McKinney et al., 1999; Richards et al., 2005; Passafaro et al., 2003). In addition, blocking AMPA/kainate receptors *in vivo* prevented estradiol-induced spinogenesis in the VMH during development, while activating them mimics estradiol-induced spinogenesis (Amateau and McCarthy, 2004; Todd et al., 2007). Therefore, initial AMPAR activation may be necessary for spinogenesis.

These ideas are not mutually exclusive, however. It is possible that AMPAR activity is necessary both fairly early after estradiol administration, as well as later, subsequent to spine formation. The former may be a downstream effect of membrane-mediated estradiol action and the latter a result of genomic estradiol mechanisms. Thus the AMPAR modulation may serve different purposes at different points in the signaling pathway. This scenario is plausible given that GluA1 immunoreactivity is decreased at one hour but increased at 76 hours plus progesterone. To test this hypothesis it must be determined when the spines first emerge.

Yet another alternative possibility is that GluA1 and GluA2 serve different purposes in the regulation of dendritic morphology. Some evidence from work in several cell types and brain areas has indicated that GluA1 may induce spine formation, while GluA2 is involved in stabilization, maintenance, or shape of dendritic spines (Prithviraj et al., 2008; Medvedev et al., 2008). Additionally, AMPAR subunits have also been associated with dendritic motility and

arborization as opposed to spine density regulation (Chen et al., 2009; Rajan and Cline, 1998; Wong et al., 2000; Haas et al., 2006).

Another interesting question our results raise is in which cells are AMPAR subunits changing? It may only occur on dendrites of estrogen receptor (ER)α-containing cells or it may be a global effect. One study cites 50% colocalization of ERα with both GluA1 and GluA2/3 in the VMH (Diano et al., 1997). However, our preliminary experiment employing fluorescent confocal microscopy indicated that in the vlVMH, all ERα cells visualized were GluA1-positive, but not all GluA1-expressing cells were ERα-positive (Fig. A.3A-C). This colocalization indicates a possible direct effect of estradiol in which it implements changes via receptors on the same cell. This is an interesting finding considering that spine increases on short primary dendrites occurred almost exclusively in non-ERα-expressing cells, suggesting a transynaptic hormone effect (Calizo and Flanagan-Cato, 2000). A similar, likely transynaptic estradiol-induced increase in spines occurs in the hippocampus as well (Cooke and Woolley, 2005). These data imply that spine changes and GluA1 changes may occur on dendrites of different cell types, another indication of the complex spatial specificity of ovarian hormone-mediated neuroplasticity.

Here we have shown GluA1 and GluA2 immunoreactivity levels respond to ovarian hormones at 76 hours. While we show a decrease in GluA2 after estradiol alone, a previous study reported an increase in both GluA1 and GluA2/3 in the hypothalamus after estradiol treatment. However, the effects of estradiol on GluA2 only in the VMH specifically were not assessed by this previous study. In addition, the previous design included supraphysiological doses of estradiol administration for an extended period of time (7 days) (Diano et al., 1997). Thus, it is difficult to interpret the contrasting results, as the experimental paradigms were quite different.

The present study did not include an examination of GluA3 and GluA4 immunoreactivity, as they have not been associated with plasticity to the extent of GluA1 and GluA2. Previous research suggests that GluA3 increases in the vlVMH in response to a supraphysiological dose of estradiol, but there is very little GluA4 present in this area (Diano et al., 1997; Eyigor et al., 2001).

It is not clear what changes we would expect to uncover in these subunits in our paradigm. In addition to unknown levels of specific AMPAR subunits, changes in total number of AMPAR in response to hormone treatment are not known. The number of receptors may increase, decrease, or remain constant while changing the ratio of subunit expression. A closer look at these details is warranted. Despite these missing details, we can speculate about the consequences of subunit variability, as presence or absence of the GluA2 subunit alone dictates the ion selectivity of the tetrameric receptor; GluA2-containing receptors are calcium-impermeable and outwardly rectifying, and GluA2-lacking receptors are calcium-permeable and inwardly rectifying (Isaac et al., 2007). Changes in calcium dynamics can have extensive effects on the molecular mechanisms of the cell and as a result, the brain nucleus. For example, calcium activates various enzymes, regulates opening of channels, and affects gene expression, which can lead to widespread and lasting changes. Calcium signaling has been shown to be important for behavior, as an inhibitor of the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) attenuated lordosis response (Balasubramanian et al., 2008). This is somewhat surprising, considering that at the time of lordosis, with estradiol and progesterone present, GluA2 is higher than in the estradiol alone condition, leading to decreased calcium permeability, and presumably decreased CaMKII activity and lordosis. Thus, future experiments should aim to determine ovarian hormone-dependent calcium signaling mechanisms.

With both estradiol and progesterone present, when the animal is at the peak of sexual receptivity and spines are increased, GluA1 and GluA2 are also at their relative maximum levels. This may seem surprising given that glutamate is inhibitory to female sexual behavior. When administered intracerebroventricularly or into the vlVMH, glutamate, AMPA, and NMDA decreased lordosis behavior, while AMPAR and NMDAR antagonists facilitated female sexual behavior (Kow et al., 1985; McCarthy et al., 1991; Georgescu and Pfaus, 2006a; Georgescu and Pfaus, 2006b; Fleischmann et al., 1991). Thus, we might expect a decrease in glutamate receptors at the time of lordosis. However, as previously mentioned, our data only considers AMPAR subunits, not total numbers of AMPAR, so investigating total AMPAR, as well as NMDAR

and metabotropic glutamate receptor expression at this time would be informative. Interestingly, evoked excitatory postsynaptic potentials (EPSPs) in the vlVMH were lowest when females were at the sexually receptive phase of their estrous cycle, when estradiol and progesterone levels are high. These EPSPs were abolished by the AMPAR antagonist DNQX, suggesting that AMPAR activity would be lower in the estradiol plus progesterone condition (Booth et al, 2010).

Besides postsynaptic changes affecting glutamate transmission, presynaptic release may also be changing. However, results have been conflicting. One study utilizing high performance liquid chromatography (HPLC) determined that estradiol increased glutamate release in the VMH, while estradiol plus progesterone decreased it (Luine et al., 1997). These data agree with behavioral experiments that demonstrate low levels of lordosis in the estradiol only, but high levels in estradiol plus progesterone. Another study employing fluorometry indicated that estradiol caused no change in glutamate release (Frankfurt et al., 1984). On the other hand, during developmental estradiol-induced spinogenesis in the hypothalamus, glutamate release occurs within six hours of estradiol administration (Schwarz et al., 2008). The effect of progesterone on glutamate release was not explored, however, and because it is responsible for a robust increase in sexual receptivity, we might expect it to cause a reduction in glutamate release in the vlVMH. Therefore both presynaptic and postsynaptic glutamate activity needs to be explored more thoroughly in future studies.

As for changes in AMPAR subunits in the amygdala, estradiol decreases GluA1 in the BLA at 30 minutes compared to vehicle treatment, and by 1 hour the expression level remains decreased, similar to that of the control at that time. A further estradiol-induced decrease occurs at 4 hours. In the CeA there was only a decrease in GluA1 at 30 minutes. As in the vlVMH, the AMPAR changes follow those in p-cofilin:cofilin. In both the BLA and CeA, GluA2 is decreased in the 76-hour estradiol condition, but restored to baseline levels with the addition of progesterone, the same pattern that occurs in the vlVMH. There is reason to believe that these changes in AMPAR subunits are important for behavior. AMPAR antagonists delivered into the BLA blocked fear-potentiated startle (Walker and Davis, 1997). It seems reasonable that ovarian hormone-

mediated changes in AMPAR in the amygdala would contribute to an anxiolytic effect that is likely necessary in order for the animal to focus on mating behavior. Preliminary observation of colocalization of ERα and GluA1 in the amygdala revealed that all BLA cells that expressed GluA1 cytoplasmically in the soma also expressed ERα (Fig. A.3D-F), although most GluA1 was present in what appeared to be dendrites. In the CeA on the other hand, there seemed to be much less colocalization (Fig. A.3G-I). These data show that different mechanisms of estradiol modulation may be taking place in these nuclei and are a promising first step for future research. It will be necessary to elucidate additional alterations in expression, morphology, and behavior that are taking place in order to interpret the consequences of the changes we have observed.

The current study has helped identify a rapid effect of estradiol in the sexual behavior-relevant brain area, which is indicative of membrane-associated estrogen receptor (ER) action. A similar effect was found in the neighboring arcuate nucleus of the hypothalamus, which displayed an increase in p-cofilin at one hour, although earlier timepoints were not examined, nor were total cofilin levels measured (Christensen et al., 2011). This same study determined that the toxin cytochalasin D was able to prevent estradiol-induced spinogenesis and as a result inhibit lordosis behavior. Interestingly, mGluR1a was necessary for the rapid increase in p-cofilin (Christensen et al., 2011). Therefore, it will be very important to determine whether any of these same mechanisms are important in the vlVMH. Similarly, an estrogen-mediated pathway identified in hippocampal slices is also worth exploring. In this system, the small GTPase RhoA is stimulated, which activates RhoA kinase (ROCK), resulting in phosphorylation of LIM kinase, which in turn phosphorylates cofilin, ending in an increase in F-actin in spines. Interestingly, these events were mediated by membrane-associated ERβ (Kramar et al., 2009). As the vlVMH does not express ERβ, only ERα, and ERα but not ERβ agonists systemically promote lordosis, it will be important to assess if any of these mechanisms occur through other estrogen receptor types in the vlVMH (Shughrue et al., 1997; Mazzucco et al., 2008). A possible mechanism may involve BDNF, which can be induced by estradiol, and its receptor TrkB has also been associated with the RhoA/ROCK/cofilin pathway (Spencer et al., 2008; Scharfman and Maclusky, 2005; Kramar et al.,

2013 for review). In another study by the same group, BDNF application, or treatment with an ampakine, a positive modulator of AMPA-like receptors known to amplify BDNF, upregulated F-actin expression in spines (Kramar et al., 2012). As these experiments were conducted with preparations from male rats and females that had been estrogen deprived for several months, is important to note that similar BDNF/LIMK/cofilin/estradiol-induced spines have also been suggested in the hippocampus of adult cycling mice (Spencer et al., 2008), and may also be involved in the vlVMH-lordosis circuit as well.

Exploration of the two estrogen pathways, membrane receptor-associated rapid effects and nuclear-receptor-associated genomic effects, is vital, as both are necessary for female sexual behavior (Kow and Pfaff, 2004). It is likely that membrane effects mediate the 30 minute changes while genomic effects regulate the variations in expression at four and 76 hours. Other candidates to investigate that may be involved in the mechanism of rapid estradiol effects include protein kinases A and C (PKA and PKC) (Kow and Pfaff, 2004). Some important tools that may be utilized to help dissociate which pathway is being activated and which receptors are involved are membrane-impermeant estrogens (E-BSA), nuclear receptor-specific blockers (ICI-182,780), selective estrogen receptor modulators (SERMs), and membrane receptor agonists (STX).

Factors to investigate in future studies have been referenced throughout this discussion. However, the overarching goal will be to demonstrate the necessity of vlVMH dendritic spines for lordosis behavior. Another critical experiment should determine the earliest timepoint at which an increase in dendritic spines is detectable. Our immunohistochemical results suggest that the estradiol-induced onset occurs anywhere between one and 76 hours. Preliminary results of our recent Golgi study suggest that the increase in spines is not yet present at four hours. We examined dendritic morphology of 5-6 vlVMH neurons per animal four or 76 hours after vehicle or estradiol treatment. Figure A.4A-C show that total number of dendrites, number of short primary dendrites, and number of secondary dendrites did not differ between treatment groups. This is an expected result, as a previous Golgi study also reported no hormone effect on dendrite number at 76 hours (Griffin and Flanagan-Cato, 2008). This same experiment revealed an estradiol-induced

decrease in length of long primary dendrites, an effect that was not replicated in the current results (Fig A.5B), although the sample sizes are very small. Additionally, no hormone effect on dendrite length was observed at four hours (Fig A.5A). Finally, our results showed a possible estradiol-induced increase in spine density on both long and short primary dendrites at 76 hours (Fig. A.6B), where a previous effect was only observed on short primary dendrites (Calizo and Flanagan-Cato, 2000), but again, the sample sizes are extremely small. Additionally, previous results were obtained in Lucifer yellow-filled cells versus the Golgi impregnation utilized here. Both techniques rely on random cell visualization, which could be unintentionally biasing. Nevertheless, there were no estradiol effects on spine density on any type of dendrite examined at four hours (Fig. A.6A). Thus, an increase in sample size at these timepoints as well as the addition of different timepoints is critical. These pilot data indicate that perhaps hormone-regulated spinogenesis is initiated with cofilin phosphorylation at 30 minutes, but structural changes are not apparent until after four hours. A close examination of spine shape will also be interesting, in addition to determination of cell type on which these changes are occurring.

A final possibility to explore is progesterone's ability to induce rapid effects. While such instances have been described, they have mostly been related to neuroprotection (Qiu et al., 2003; Kuo et al, 2010).

Conclusion

Our current data reflect rapid, transient estradiol-mediated changes in the ratio of p-cofilin:cofilin within 30 minutes in the vlVMH and LFC, which is likely the result of membrane receptor action. Changes in p-cofilin:cofilin increase one hour after estradiol in the BLA and CeA, but GluA1 was decreased at 30 minutes in both areas. At four hours, the vlVMH, dmVMH, and BLA all reflect an estradiol-induced decrease in GluA1 density. By 76 hours post-estradiol, when increased spine density has been observed, GluA2 is decreased in the vlVMH, BLA, and CeA. The addition of progesterone results in increased GluA1 in the vlVMH and LFC. A summary of our results is represented in Table 4.1. Here, we have shown dynamic and spatially-specific

changes in protein expression in various brain areas over a wide timecourse that likely contribute to the mechanism underlying ovarian hormone-regulated spine changes and female sexual behavior.

Time	vlVMH			dmVMH			LFC			BLA			CeA		
	ratio	G1	G2	ratio	G1	G2	ratio	G1	G2	ratio	G1	G2	Ratio	G1	G2
0.5	↑	-	X	-	-	X	↑	-	X	-	↓	X	-	↓	X
1	-	-	X	-	-	X	-	-	X	↑	-	X	↑	-	X
4	-	↓	X	-	↓	X	-	-	X	-	↓	X	-	-	X
76	-	-	↓	-	-	-	-	-	X	-	-	↓	-	-	↓
76 + P	X	↑	-	X	-	-	X	↑	X	X	-	-	X	-	-

Table 4.1. Summary of relative protein levels over time after estradiol or estradiol plus progesterone (P) administration.
All results reflect changes significant from their respective vehicle groups. Ratio = p-cofilin:cofilin levels; G1 = GluA1; G2 = GluA2; vlVMH = ventrolateral subdivision of the ventromedial nucleus of the hypothalamus; dmVMH = dorsomedial subdivision of the VMH; LFC = lateral fiber complex; BLA = basolateral nucleus of the amygdala; CeA = central nucleus of the amygdala. ↑ = an increase, ↓ = a decrease, - = no change compared to vehicle, X = not measured.

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CHAPTER 5: General Discussion

Estradiol and progesterone cause a number of structural and morphological changes throughout the brain. These phenomena likely affect numerous behaviors, but many of the causes and effects of hormone-induced neural plasticity are yet unexplored. Even just within the ventrolateral subdivision of the ventromedial nucleus of the hypothalamus (vlVMH) and neighboring lateral fiber tract (LFC), ovarian hormones stimulate several changes that coordinate to prepare the brain and body for reproduction. This thesis specifically focused on ovarian hormone-induced changes in dendrite length and spine density.

Species Comparisons

In Chapter Two, I determined that dendrite length is correlated to mating status in female prairie voles, as it is in female rats. Considering the vast differences in reproductive systems and sexual behavior in the two species, this finding of evolutionary conservation highlighted the importance of such a structural change. Rats are polygynandrous, exhibit maternal parenting, and experience spontaneous estrous and ovulation, while female prairie voles are monogamous, engage in biparental care, and undergo induced estrous following cohabitation with a male and induced ovulation following mating (Carter and Getz, 1993; Carter et al., 1980; Curtis, 2010; Witt et al., 1988). However, in both species, estradiol levels, reproductive behavior, and dendrite length were related. In previous studies of dendrite morphology in rats, exogenous hormone treatment after ovariectomy (OVX) clearly demonstrated that estradiol and progesterone caused changes in dendrite length. By exposing female prairie voles to males in order to stimulate estradiol secretion in this study, we are not able to definitively determine the direction of causation, but this paradigm allowed us to discern divergent behaviors--reproductively activated versus not--and yielded hormone-associated length differences nevertheless.

In rats, naturally cycling females have longer dendrites during proestrus, when estradiol and progesterone levels are elevated and lordosis expression is at its peak, than during diestrus, when ovarian hormones are low (Madeira et al., 2001). In OVX rats, estradiol administration decreases the length of long primary dendrites in the vlVMH, whereas estradiol plus progesterone increases long primary dendrite length (Griffin and Flanagan-Cato, 2008). In a study with a similar experimental paradigm, in OVX Syrian hamsters, estradiol had the opposite effect of increasing dendrite length, although it was expressed as total dendrite length rather than identifying dendrite type. In that analysis, estradiol plus progesterone had no further effect on dendrite length, which was significantly longer than in the vehicle condition (Meisel and Luttrell, 1990). Finally, as described here previously, in prairie voles, exposure to a male that resulted in reproductive activation, including robust receptive behavior and increased plasma estradiol levels, was related to increased length of long primary dendrites in the vlVMH compared to females that did not become reproductively activated. While these data may seem discrepant, rats and Syrian hamsters need both estradiol and progesterone in order to exhibit sex behavior, but prairie voles do not. We did not investigate the effects of progesterone on the dendritic tree in voles because it is not necessary for lordosis display in this species, and is not secreted until after mating occurs. Thus, in all three species, while variations in timing and likely in molecular mechanisms exist, females are receptive when dendrites are extended.

It is important to note that in the previous study in which OVX rats were administered exogenous hormones, the long primary dendrites (LPDs) in the vehicle condition were as long as those in the estradiol plus progesterone condition (Griffin and Flanagan-Cato, 2008). Similarly, in Chapter Two, I showed that while reproductively activated voles (RA) had significantly longer LPDs than non-reproductively activated females (NA), unpaired voles (UP) had long primary dendrites that were intermediate. Both rats in the vehicle group and voles in the UP group would exhibit little to no sexual receptivity under these conditions, yet have relatively long LPDs. Thus dendrite length is clearly not the only factor necessary for lordosis behavior. Neurochemical changes are also critical, such as expression of progesterone receptors and oxytocin receptors

(see Flanagan-Cato, 2011 for review). In addition, numerous other factors, including glutamate activity, spine density changes on short primary dendrites and PAG-projecting neurons, numbers of synapses, AMPAR subunit expression, would not be properly expressed or positioned to support lordosis behavior (Georgescu and Pfaus, 2006a; Georgescu and Pfaus, 2006b; Booth et al., 2010; Calizo and Flanagan-Cato, 2000; Calizo and Flanagan-Cato, 2002; Carrer and Aoki. 1982; Ferri and Flanagan-Cato, unpublished observations, see chapter 4). In the case of the unpaired female prairie voles, far fewer data pertaining to the role of the vlVMH in mating behavior are available, but given their similar dendritic morphology, we might expect a similar lack of estradiol-induced modifications necessary for reproductive behavior, regardless of dendrite length. Therefore, many prerequisites must be fulfilled prior to induction of female sexual behavior, but contingent upon some of these factors, long LPDs are an important part of the lordosis circuit.

Dendrite length and mating status

The preserved dendritic tree in rats and prairie voles implies a necessity of maximal extension of long dendrites for facilitation of mating. Our lab has proposed that because most afferents do not project into the vlVMH proper, but are restricted to the area of the lateral fiber complex (LFC), dendrite elongation is likely the primary mechanism of regulation of extranuclear input (Millhouse, 1973a; Griffin and Flanagan-Cato, 2008; Griffin et al., 2010). The proximity of these fibers and their inclusion of neurotransmitters and neuromodulators, such as norepinephrine and oxytocin, which are important for female sexual behavior, are provocative (Millhouse, 1973; Etgen et al., 1992; Schumacher et al., 1990; Griffin et al., 2008). The hypothesis was strengthened by the demonstration of synapses in the LFC, including glutamatergic and oxytocinergic synapses among dendrites extending from the vlVMH and axonal boutons from the adjacent fibers (Griffin et al., 2010).

Evolutionarily, a sound rationale for such complex changes to occur every 4-5 days over the estrous cycle is to conserve energy long-term, or at least redirect it to other activities. Such a

mechanism would help to minimize time wasted in reproductive efforts during infertile times. Instead, energy is best utilized obtaining food or building a nest for future offspring.

The conservation of this phenomenon in prairie voles, which exhibit the unusual behavior of monogamy (reviewed by Curtis, 2010) is important, as it suggests shared mechanisms of mating control in other species (Griffin and Flanagan-Cato, 2008; Flanagan-Cato, 2011). Furthermore, the relation of dendrite length to mating status can begin to provide insight into infertility and/or mate preference. The observation that approximately 25% of female prairie voles typically do not respond to initial male exposure raises many interesting questions, and several non-mutually exclusive possibilities. It is conceivable that the failure of females to become reproductively activated after cohabitation with a male is a reflection of the female's determination that the male is not a suitable mate. Major histocompatibility complexes (MHC) are molecules that mediate immune functions, but also aid in the olfactory detection of identity and immune function of conspecifics, which contributes to mate selection processes in fish, mice, primates, and humans (Milinski et al., 2005; Spehr et al., 2006; Havlicek and Roberts, 2009; Jacob et al., 2002). Thus, it is possible that these factors signaled incompatibility to the female due to an immune function exceedingly similar to her own, which would decrease genetic diversity and result in offspring vulnerability (Penn and Potts, 1998). While the degree of involvement of MHC in prairie vole mating is not well defined, these complexes may play a role in the interaction between dendrite length and estradiol. The contribution of MHC to female receptivity would not be surprising, as prairie voles are highly dependent upon pheromones for pair bonding and mating, and are particularly effective at avoiding inbreeding (Carter and Getz, 1993; Pusey and Wolf, 1996; Gavish et al., 1984). On the other hand, mate incompatibility and naturally occurring infertility are not well described, and rats are more likely to engage in inbreeding than some other rodents. Therefore, it would be interesting to investigate whether they, and other species, exhibit shorter long primary dendrites in the vlVMH when they display reduced willingness/desire to mate or ability to reproduce.

Another possibility that may help reduce the percentage of females that do not become reproductively activated is allowing them more cohabitation time with the male. This may override the non-preferred status of the male's MHC, or it may provide the extra time needed to produce sufficient male pheromone-induced estradiol to allow extension of long primary dendrites. This option is plausible because male contact resulted in a correlation of estradiol levels with dendrite length in reproductively activated animals and non-reproductively activated animals, but not in the unpaired females, which points to regulation of the factors by interaction with a male. Examining some of these possibilities may help provide some insight into infertility, mate choice, and sexual dysfunction.

The LFC and the role of OT

After extending VMH dendrite regulation to another species, Chapter Three further characterized the LFC, the important area in which the evolutionarily conserved pattern of dendrite extension occurs. An effective method suited to investigate ultrastructural characteristics and synaptic reorganization in the LFC is electron microscopy. Thus, we returned to the rat model, which allows manipulation of ovarian hormones. Although electron microscopy is limited to fairly small areas of observation, it permits examination of cellular details to ascertain what types of reconfiguration occurs in the LFC in response to the dynamic hormone-induced dendrite length changes.

The fiber field adjacent to the VMH (LFC) was first described by Millhouse (1973), who observed that the VMH was encapsulated by fibers. Based on observations made in Golgi impregnated tissue, he described dendrites extending out of the VMH but few afferents entering the nucleus. He therefore suggested that axons from the fiber plexus likely form synapses with dendrites of VMH neurons. Since then, few studies have focused on the LFC. Confocal microscopy revealed a close apposition of vlVMH dendrites with oxytocin-labeled fibers in the LFC (Daniels and Flanagan-Cato, 2000), further strengthening Millhouse's hypothesis. However,

Griffin et al. (2010) first clearly exhibited synaptic contact there and provided a thorough ultrastructural characterization, which led to the follow-up analyses contained in Chapter Three.

In addition to describing the ultrastructure of the LFC and how it is affected by ovarian hormones, we investigated a local neuromodulator of lordosis located in the area. Norepinephrine, serotonin, and oxytocin are among molecules found within fibers of the LFC that influence female sexual receptivity and may also serve as possible synaptic markers. We focused on oxytocin (OT) because it is an evolutionarily conserved neuropeptide associated with numerous social behaviors including lordosis (Lee et al., 2009; Ross and Young, 2009 for review).

Interestingly, OT is well studied within prairie voles because it plays an important part in pair-bonding mechanisms. In virgin female prairie voles during a 6-hour cohabitation period with a male, intracerebroventricular administration of OT induces a partner preference, which usually requires either an extended period of cohabitation or mating during that time (Williams et al., 1994). An OT antagonist inhibits the formation of pair bond although it does not interrupt mating (Insel et al., 1995). Although rats do not exhibit pair-bonding, OT is still important in their brief sexual bond. As discussed previously, OT facilitates lordosis and antagonists attenuate it (Arletti and Bertolini, 1985; Vincent and Etgen, 1993; Caldwell et al., 1986; Benelli et al., 1994; Witt and Insel, 1991; Pedersen and Boccia, 2002).

Likewise, researchers are exploring OT's implications in human sexual behavior, mate bonding, and romantic relationships. As in animals, OT seems to have a stronger effect in females. In women, OT levels are positively correlated with inclination to express and share emotions and general feelings of attachment (Tops et al., 2007). OT release is also related to women's nonverbal displays of romantic love (Gonzaga et al., 2006). Interestingly, pretreatment with intranasal OT increases positive interactions during a conflict conversation in heterosexual couples compared to couples not receiving OT. Those in the treatment group showed more positive body language, eye contact, and self-disclosure (Ditzen et al., 2009). During sexual stimulation and arousal, circulating OT levels increase and peak during orgasm in both men and

women (Carmichael et al., 1987). This release during sex may serve to help human partners (and other species) form a bond (Ross and Young, 2009). Finally, women given intranasal OT report increased feelings of arousal (Anderson-Hunt and Dennerstein, 1994). Clearly, the human interactions represent a complex mating structure that includes an emotional element, while in many species this bond is short-lived and for reproductive purposes only, but it is nonetheless a significant social interaction. Therefore, OT is an essential element in the sexual behavior of both humans and animals.

Given that OT has a widespread importance in the sexual behavior of many species regardless of mating system, our findings that it is a marker for dendrites in the vlVMH that remain after estradiol exposure and receive increased synaptic input are an important first step in describing how it works with estradiol to promote lordosis. Previous to this discovery of OT+ dendrites and synaptic contact with LFC fibers, a bulk release model was preferred in which OT is diffused to far-away targets from its site of synthesis in the PVN and SON (Morris and Pow, 1991; Sabatier et al., 2007). An alternative hypothesis also involved diffusion, but from neurohypophyseal axons projecting to the posterior pituitary. While we have not discounted these possibilities we have provided evidence for point-to-point communication in the LFC that allows behavioral specificity. Additionally, because of the synaptic position of OT and likelihood of an OT+ dendrite being innervated by an OT+ bouton, we propose that OT is internalized with its receptor into dendrites. However, future endeavors should aim to test that hypothesis directly, perhaps with endosomal markers, to elucidate how these mechanisms work.

Thus far, suggested mechanisms of OT-ovarian hormone interaction have been incomplete. For example, there have been arguments that OT is moderated by estradiol, or that it is a moderator of estradiol. Evidence that estradiol is more important for OT function than progesterone includes the observation that exogenous OT elicits activity in estrogen-treated ventromedial hypothalamic neurons while the addition of progesterone *in vitro* caused little further effect on responses (Kow et al., 1991). Additionally, it has been proposed that the lordosis response after OT administration was similar regardless of whether or not progesterone was

present (Caldwell, 1986; Whitman and Albers, 1995). Finally, OTR antisense-oligo infusion effectively blocked lordosis, but only when females were primed with estrogen alone, not progesterone. However, this method only provided a 31% decrease in OTR binding (McCarthy et al., 1994).

A 5'-flanking region of the OT gene and an upstream palindromic estrogen response element (ERE) have been identified that are estrogen-responsive and may explain the ability of estradiol to induce OT and OTR gene transcription (Peter et al., 1990; Adan et al., 1991). Importantly, Devidze (2005) found that 86.2% of OT receptor-expressing vlVMH cells also express ERα in intact females, while estradiol-treated OVX females showed 81.8% coexpression. This finding serves as evidence that estradiol and oxytocin may both act upon the same cell and that cooperation with estradiol may be more important than interaction with progesterone.

On the other hand, some groups have reported the inability of OT to facilitate lordosis in animals treated acutely or chronically with estradiol alone (Gorzalka and Lester, 1987). Acute or chronic estradiol treatment failed to statistically increase OT mRNA in the VMH (Chung et al., 1991). By keeping the doses of estradiol and OT constant but varying the quantity of progesterone, one group concluded that the facilitation of lordosis in ovariectomized rats by centrally-administered oxytocin is progesterone-dependent (Gorzalka and Lester, 1987). Furthermore, it has been implied that progesterone is a necessary element for the lordosis pathway, but its production by the adrenal glands may be induced by relatively high levels of estradiol when the ovaries are absent. That occurrence, if true, could explain other groups' ability to induce lordosis with estradiol and oxytocin alone. High enough OT doses in conjunction with estradiol may be sufficient as well, as lordosis was observed in such circumstances, but not if adrenalectomy was performed in addition (Schumacher et al., 1992). Finally, the capacity for an OT antagonist to eliminate the lordosis reflex is only achieved when the antagonist is applied prior to progesterone administration. Four hours after the OTR antagonist and P were injected, however, lordosis quotients were reduced by 50%, and at 6 hours by 75% (Witt and Insel, 1991).

Therefore whether and how OT interacts with estradiol and/or progesterone remains

unclear. Progress toward a defined mechanism is a future goal. However, our working model may initially suggest that estradiol is a more important modulator of OT because it results in increased synaptic input to OT+ dendrites, which are likely OTR+ dendrites. However, the types of input are not clearly more or less likely to be OTergic themselves, and in fact may be glutamatergic and have a mildly inhibitory effect on behavior. Therefore, progesterone, which seems to have the effect of expanding receptors into the area of peptide release, may be more important for the ability of OT to facilitate lordosis behavior.

The effector protein kinase C (PKC) may be involved in OT modulation. Audioradiographs have shown that the PKC inhibitor BIM significantly decreased OTR binding levels in the VMH. It also blocked lordosis response in behavioral quantifications. Manipulations of protein kinase A had no effect on OTR binding or sexual behavior (Bale et al., 2001). Subsequently, a subset of vlVMH neurons was reported to coexpress ER, OTR, and one of several isoforms of PKC (Devidze et al., 2005). Finally, OTR are GPCR that have been associated with Gαq signaling, which often involves PKC (Gimpl and Fahrenholz, 2001). Therefore, the mechanism responsible for lordosis behavior may include interactions of a combination of multiple peptides, neurotransmitters, and effectors.

Further investigation into other molecular markers of synaptic plasticity in the LFC is an important next step. As previously mentioned, serotonin and norepinephrine are also contained within the LFC and both affect lordosis. Serotonin is inhibitory to the behavior, while norepinephrine is facilitatory (Zemlan, 1973; Vincent and Etgen, 1993). In fact, in the presence of estradiol and progesterone, but not estradiol alone, OT administration increased the release of norepinephrine (Vincent and Etgen, 1993). Therefore, it is reasonable to hypothesize that norephinephrine and possibly serotonin are targets of OT action.

OT and glutamate

Glutamate is likely an important factor in the OT-lordosis circuit. As Griffin's ultrastructural study revealed (2010), 100% of OT-containing axonal boutons in the LFC were

glutamatergic, as evidenced by the colocalization with VGLUT2 and the presence of small vesicles (Griffin et al., 2010). Thus, glutamate may modulate oxytocinergic activity or vice versa. Glutamate causes dendritic OT release via non-NMDA receptors (de Kock et al., 2004; Pampillo et al., 2001). Additionally, kainic acid causes a significant increase in c-Fos expression in OTergic neurons in the PVN and SON (Zehra Minbay et al., 2006). Finally, electrical activity in the SON is mediated by non-NMDA receptors (Kombian et al., 1997; Curras-Collazo et al., 2003). In contrast, OT reduces potassium-stimulated glutamate release and inhibits presynaptic release of glutamate in the SON (Moos et al., 1997; Nissen et al., 1995). OT in presynaptic boutons of the dorsal vagal complex selectively increases the probability of glutamate release (Peters et al., 2008). Also, in the PVN and SON, OT and glutamate are co-released (Hrabovszky et al., 2006 and Ponzio et al., 2006).

Behavioral studies show that OT facilitates, while glutamate inhibits, female sexual behavior (Georgescu and Pfaus, 2006a, 2006b; McCarthy et al., 1994). Peptides often serve as modulators of neurotransmitters with which they colocalize; we propose that in the vlVMH, OT modulates glutamate action (Eva et al., 2004). Specifically, the increased synaptic input on long primary dendrites after estradiol administration could reflect increased glutamate transmission, attenuating lordosis, while the possible subsequent progesterone-induced decrease in glutamate would facilitate lordosis (Luine et al., 1997). Thus, future studies are needed to test this hypothesis.

Dendrite Length

As for changes in dendritic length specifically, glutamate has been implicated in these mechanisms as well. In isolated motoneurons from embryonic rats, glutamate reduced the number of neurites and inhibited dendrite, but not axon, growth via non-NMDA ionotropic receptors. In particular, calcium influx through non-GluA2 AMPA receptors and/or kainite receptors was necessary (Metzger et al., 1998). Another mechanism proposed by Griffin and Flanagan-Cato (2011) suggests the involvement of the calcium-dependent protease calpain. In

hippocampal neurons, calpain has been shown to decrease levels of MAP2, a cytoskeletal protein specific to dendrites, after persistent stimulation of glutamate receptors (Siman and Noszek, 1988). Therefore, this protein, along with glutamate activity, may be involved in estradiol-induced dendrite retraction in the vlVMH.

A number of proteins and compounds have been related to dendritic length changes and warrant exploration. For example, levels of the homeodomain protein Cut correlate with dendritic arborization and complexity in embryonic and larval *Drosohphila* (Grueber et al, 2003). Some other molecules that may be involved in the mechanisms underlying dendrite length changes include microtubule-associated proteins (MAP), c-Jun N-terminal kinase 1 (JNK1), Rho GTPases, the glycoprotein Wnt7b, phosphoprotein Dishevelled, and growth factor bone morphogenetic protein (BMP) (Mattson, 1994; Bjorkblom et al., 2005; Rosso et al., 2004; Withers et al., 2000). In addition, a synthetic peptide that corresponds to the β-amyloid protein increased dendrite-like processes and their arborization in hippocampal cultures (Whitson et al., 1990). Also, several mRNAs that provide the machinery for protein synthesis that permits dendrite outgrowth were identified in dendritic growth cones of hippocampal neurons (Crino and Eberwine, 1996). Thus, while there are a number of candidates implicated in dendrite growth mechanisms, most of these are associated with dendrite and neurite outgrowth during development, in other brain areas, or in cell cultures. It is not clear whether similar factors are involved in changes that occur during adulthood, in the hypothalamus *in vivo*.

Similarly, proteins such as Slit and Robo, the neuronal kinase cyclin-dependent kinase 5 (Cdk5), and various neurotrophic factors have been identified that are involved in axon guidance (Marillat et al., 2002; Ye et al., 2012; Harvey et al., 2012). Interestingly, the cytoplasmic- and membrane-associated axonal protein GAP-43 implicated in elongation is regulated in the vlVMH by estradiol levels of intact and hormone-treated OVX females (Lustig et al., 1991; Shughrue and Dorsa, 1993; Sa and Madeira, 2012). Serotonin has been shown to suppress neurite outgrowth, which is complementary to its suppressive effect on female sexual behavior (Trakhtenberg and Goldberg, 2012). These factors have been implicated in different organisms, brain areas, and

stages of development. Some of the same molecules associated with growth of axons may also affect dendrite growth.

Finally, brain-derived neurotrophic factor (BDNF) is likely involved in the neural plasticity exhibited by the LFC. BDNF has been associated with estradiol-induced dendrite growth in the cerebellum (Sashahara et al., 2007). BDNF mRNA, protein, and its receptor, TrkB, have been localized to the VMH, and the mRNA is regulated by estradiol levels (Conner et al., 1997; Sugiyama et al., 2003; Tran et al., 2006; Zhu et al., 2013). However, to date, most research involving BDNF action in the VMH has been focused on feeding behavior within the dorsomedial subdivision. It will be important to investigate its effects and mechanisms in the vlVMH specifically, and its association with reproductive behavior.

Some final thoughts to consider relate to implications of the synaptic reorganization described here. We have proposed that the purpose of the dendrite length changes is to make contact with the LFC to regulate levels of afferent input, which is also supported by changes in axonal innervation. This connects dendrite length alterations to variable input, but it can also be related to output as well. CA3 pyramidal cells with longer dendrites were more likely to exhibit burst firing, likely due to ion channel distribution (Bilkey and Schwartzcronin, 1990). On the other hand, even when controlling for ion channel distribution, a model of neocortical neuron firing suggests that the dendritic geometry alone correlates with firing pattern (Mainen and Sejnowski, 1996). Currently, these dynamics remain unexplored in the vlVMH.

Other implications of changes in dendritic morphology include pathology-associated deficits. Patients with schizophrenia and patients with Alzheimer's disease exhibit smaller dendritic trees of neurons in layer V pyramidal neurons or CA1 pyramidal cells, respectively (Black et al., 2004; Yamada et al., 1988). Likewise, autism spectrum disorders have been associated with dendritic branching abnormalities (Pardo and Eberhart, 2007). In addition, 25 days of electrically elicited seizures results in a short-term increase in dendrite length and branching in neocortical layer V pyramidal neurons, but an overall decrease three weeks later. These results indicate that dendritic morphology has important implications on the way cells in

that brain area function, which can result in significant behavioral effects. Within the vlVMH, dendritic configuration is important for female sexual behavior, as evidenced by mating deficits in prairie voles with shorter dendrites and changes in synaptic input that correlate with female rats' behavior.

To summarize, OT plays an important role in the cellular reorganization of the vlVMH and LFC, which entails an estradiol-induced retraction of dendrites and results in increased synaptic input of dendrites that likely express OTR and internalized peptide (see Fig. 5.1). Estradiol-induced OT transmission in turn may result in an increase in glutamate release in order to inhibit lordosis behavior until progesterone is secreted, resulting in lengthened non-OT dendrites and the expression of sexual behavior. However, controlled and precise glutamate signaling within the confines of the VMH and LFC may not have the inhibitory effects on behavior as when the areas are overpowered by pharmacological intervention. This idea was discussed in Chapter Four. While questions remain regarding the mechanisms involved and further consequences of these changes, the present data demonstrate the complex and dynamic specificity of actions and connections involved in the lordosis circuit.

Dendritic spine mechanisms

Another example of spatial specificity exhibited by dendrites of vlVMH neurons is the ovarian hormone-regulated expression of dendritic spine density. As previously discussed, dendritic spine density increases on short primary dendrites, while long primary dendrites of cells with axonal projections to the PAG exhibit an estradiol-induced decrease in spine density (Calizo and Flanagan-Cato, 2000; Calizo and Flanagan-Cato, 2002). Such intricate changes likely demonstrate an important temporal specificity as well. Therefore, in Chapter Four, I addressed a possible timecourse and proteins involved in the underlying mechanism for such spine changes.

Estradiol increases the p-cofilin:cofilin ratio in the vlVMH and LFC within 30 minutes and may be an early step in the formation of hormone-induced spinogenesis. While it is uncertain when the spines first emerge, studies have shown that they are present 76 hours after estradiol

administration as well as after the addition of progesterone, when lordosis would also be observed. At this later timepoint estradiol decreases GluA2 and estradiol plus progesterone increase in GluA1. Thus, we hypothesize that reconfigured AMPAR are inserted into new spines.

Additionally, an interesting pattern was discovered in the amygdala that was different from that observed in the hypothalamus. In the BLA and CeA, rapid effects were detected in AMPAR subunit expression and changes in relative p-cofilin occurred in these nuclei after those in the vlVMH and LFC. Spine morphology in response to ovarian hormones in these areas remains unexplored and will be addressed in future studies, but our results indicate for the first time that spine changes in the BLA and CeA may occur, and likely take place after hypothalamic spine changes. Again, this is interesting given that our data indicate an increase in spines while the anxiolytic effects of estradiol would dictate a probable decrease (Mitra et al., 2005; Frye and Walf, 2004). Estradiol's anxiolytic effects seem particularly important in a lordosis-inducing paradigm, as a female is less likely to mate when enduring high levels of stress. In addition, the amygdala expresses ERα and ERβ whereas the vlVMH expresses only ERα (Shughrue et al., 1997). This observation, as well as the differences in expression over time, indicates different mechanisms are likely utilized. Finally, it will be interesting to expand research regarding rapid changes in GluA1, as both the input and output of the BLA, which relays information to the CeA, are glutamatergic (LeDoux, 1998).

These findings uncovered some specific timepoints and crucial molecules involved in estradiol-induced spinogenesis; however, many details of the mechanism remain uncertain. In the hippocampus, estradiol-induced increases in spines are fairly well characterized and thus provide valuable clues to mechanisms that may also apply to the vlVMH. This is especially true, considering some similarities in the patterns of plasticity. In both the vlVMH and the hippocampus, spine changes in response to estradiol occur transynaptically, on cells that do not express estrogen receptors (Calizo and Flanagan-Cato, 2000; Calizo and Flanagan-Cato, 2002; Weiland et al., 1997; Hart et al., 2001). Likewise, in the hippocampus, estradiol increases the number of presynaptic boutons that synapse with multiple dendritic spines, similar to the synaptic

reorganization described in Chapter Three (Woolley et al., 1996). Thus, it is possible that vlVMH shares some mechanistic characteristics with the RhoA, ROCK, LIM kinase, p-cofilin pathway that occurs in estradiol-induced spinogenesis on hippocampal neurons (Kramar et al., 2009; discussed in Chapter Four). However, as mentioned previously, that mechanism is ERβ-dependent, and the vlVMH contains only ERα (Kramar et al., 2009; Shughrue et al., 1997).

Several other mediators of hormone-dependent spine formation have been suggested as well. Gamma-aminobutyric acid (GABA) is one neurotransmitter that may be involved in spine dynamics. Based on additional studies performed in the hippocampal cultures, GABAergic input to spiny neurons is inversely correlated to subsequent estradiol-induced increases in spine density (Murphy and Segal). Electrophysiological experiments reinforce this effect via decreased inhibitory postsynaptic currents (IPSCs) before estradiol-induced spinogenesis (Rudick and Woolley, 2001; Rudick et al., 2003). This decrease in GABA activity reportedly results in an increase in excitatory synaptic activity and cAMP response element binding protein (CREB) activation, which facilitates estradiol actions (Murphy and Segal, 1997). GABA immunoreactivity and GABA-A receptors can be found in the VMH as well (Commons et al., 1999; Grattan and Selmanoff, 1997). Spontaneous inhibitory postsynaptic currents (sIPSCs) have been found in presynaptic neurons localized in this area (Jang et al., 2001).Interestingly, agonists of these receptors result in promotion of sexual behavior (McCarthy et al., 1990), whereas GABA-A receptor antagonists diminish sexual behavior (Roy et al., 1985). Thus, the seemingly contradictory inclination of GABA to decrease spines but facilitate lordosis at a time of increased spines will require further investigation.

Relatedly, in the hippocampus, cholinergic subcortical inputs may be necessary for disinhibition and estradiol-induced spinogenesis (Cooke and Woolley, 2005; Leranth et al., 2000; Lam and Leranth, 2003; Rudick et al., 2003). Acetylcholine (Ach) also facilitates lordosis, but its role in hormone-related spine formation is not known (Clemens et al., 1981; Kaufman et al., 1988; Clemens et al., 1989). Therefore, it will be interesting to determine if the increase in lordosis

behavior by these neurotransmitters is related to their effects on increased spines in the presence of estradiol and progesterone and how cofilin dynamics are involved.

Role of AMPAR

Interestingly, a recent study has shown that in order for AMPAR to be inserted into spines, an increase in cofilin activity must precede an increase in the phosphorylated form. The results uncovered a temporal specificity in which a rapid, transient decrease in the ratio of phosphorylated to total cofilin is followed by amplification of p-cofilin:cofilin and then AMPAR addition. This sequence was in response to chemically-induced LTP (Gu et al., 2010), but it may also occur within our model of study. Such a mechanism may explain why there was a significant effect of time on cofilin levels in all brain areas analyzed regardless of hormone group, wherein levels were highest at 30 minutes post-injection. While I did not observe a decrease in p-cofilin:cofilin ratio and the pattern of early increased cofilin occurred regardless of treatment, perhaps examination of earlier timepoints would reveal such an occurrence.

As already discussed in Chapter Four, brain-derived neurotrophic factor (BDNF) likely plays a role in hormonal regulation of dendritic spine density. BDNF can be induced by estradiol, and its receptor TrkB has also been associated with the RhoA/ROCK/cofilin pathway and an increase in F-actin in the hippocampus (Spencer et al., 2008; Scharfman and MacLusky, Kramar et al., 2008; Kramar et al., 2013 for review). Again, BDNF and its receptor are expressed in the VMH, and BDNF mRNA is modulated by estradiol (Conner et al., 1997; Sugiyama et al., 2003; Tran et al., 2006; Zhu et al., 2013). It is possible that estradiol transactivates the TrkB receptor, facilitates BDNF action, or causes BDNF release in the vlVMH as has been suggested in other brain areas (Lee and Chao, 2001, Nagappan et al., 2008; Huang and McNamara, 2010; Krizsan-Agbas et al., 2003, Scharfman et al., 2003 and Sato et al., 2007).

Calcium dynamics have already been discussed as possibly influencing dendrite length, but they can also be influenced by spine formation and AMPAR action. As previously discussed, AMPAR subunits determine the calcium permeability of the pore. Importantly, increases in

postsynaptic calcium facilitate actin branching and subsequent spine enlargement (Lee et al., 2012). Thus, if the current study had included spine size measurements after the induction of AMPAR we might expect to observe larger spine heads. Likewise, CaMKII contributes to spine stability by direct interaction with actin filaments, and has been associated with stimulation of Rho-GTPases that have been indicated in spinogenesis (Lee et al., 2012). Thus, CaMKII may be associated with a shift to increased mushroom spines in the vlVMH after estradiol and progesterone's induction of AMPAR. Again, in the estradiol alone condition, GluA2 is decreased, resulting in an increase in calcium permeability and a likely increase in CaMKII, which might be expected to increase lordosis behavior (Isaac et al., 2007; Balasubramanian et al., 2008). However, that is not the documented effect; estradiol without progesterone induces only very low levels of lordosis. Thus, calcium likely has different consequences at different times during hormone-induced plasticity. Therefore, calcium dynamics in the signaling mechanisms underlying lordosis warrant further investigation.

Other factors involved in spinogenesis

Finally, there are a multitude of other proteins and molecules associated with spine formation. Interactions of any of these candidates with estradiol, cofilin, or AMPAR will be important to determine in the future. A few of these proteins include Shank and Homer, which are related to NMDAR and mGluR complexes in the postsynaptic density, Sept7, a GTP-binding protein, spinophilin, a protein that interacts with actin and protein phosphatase-1, the fragile X mental retardation protein, and protein kinase B (Akt) (Sala et al., 2001; Xie et al., 2007; Feng et al., 2000; Comery et al., 1997; Spencer et al., 2008). In addition to determining if and how these molecules are associated with the lordosis circuit, timing of involvement would also be interesting.

Like dendritic length and branching, dendritic spine morphology has important repercussion on how cells function. These main sites of excitatory input integrate information which can then influence output. Spines exist in a variety of sizes, including variable spine head width and neck length, and shapes, including filopodial, thin, stubby, and mushroom. These

characteristics enable functional variety and provide information about their dynamics. For example, spine size is correlated with synapse strength. Thin and filopodial spines are associated with plasticity, and mushroom spines are correlated with stability (Rochefort and Konnerth, 2012; Lee et al., 2012). In addition, AMPAR are more likely to be expressed on mushroom spines than thin or filopodial spines (Matsuzaki et al., 2001). Our analysis did not assess spine shape or size, but this information will provide crucial indications of how the spines likely function, and how those features change over time in response to ovarian hormones.

Also similar to the importance of dendrite length, deficits in spine number or formation can have serious consequences. Mental retardation and related disorders such as Down's syndrome, Fragile X syndrome, and Rett syndrome are all associated with malformation of dendritic spines (Kaufmann and Moser, 2000). Interestingly, disruption of cofilin also causes serious deficits, and has been linked to Alzheimer's disease, HIV, and cancer. Additionally, knockout of the cofilin-1 gene in mice is embryonic lethal (see Bernstein and Bamberg, 2010 for review).

In summary, Chapter Four begins to identify some of the important factors involved in ovarian hormone-induced spinogenesis in the vlVMH as well as information about how rapidly some of these changes occur. Other vital questions that remain will include study of timepoints earlier and intermediate to those chosen here and investigation of spine morphology, including time of induction, size and shape analysis, and characterization of cell populations and dendrite types on which the changes occur. Additionally, it is important to identify the estrogen receptors involved and their locations—nuclear versus membrane-associated—and to determine whether spines in the vlVMH are necessary for lordosis behavior.

Conclusions

Integrating the data from these chapters leaves us with an updated working model of vlVMH cell types (see Figure 5.1 compared to Figure 1.1). We propose that one vlVMH cell type expresses nuclear ERα and has long primary dendrites that contain OT, express OTR, and are

innervated by several LFC axonal boutons. These cells respond to estradiol by upregulating progesterone receptors in the nucleus and OTR in dendrites, and the long primary dendrite remains extended and receives increased input (Fig. 5.1A). Figure 5.1B indicates that one subset of non-ERα-expressing cells have long primary dendrites that retract in response to estradiol and receive less innervation from the LFC than ERα-expressing cells. Two other non-ERα-expressing cell types are represented in Figure 5.1C and D. One exhibits an estradiol-induced decrease in dendritic spines on long primary dendrites on cells that have PAG-projecting axons and the other exhibits an estradiol-induced increase in spines on short primary dendrites. However, it is possible that some of these three non-ERα-expressing cell types overlap and that there may other cell types not yet described.

Although some assumptions involved require confirmation, a tentative but spatially and temporally specific configuration of the vlVMH emerges as well. In the early stages of estradiol treatment relative to a control condition, the relative amount of phosphorylated cofilin rises, enabling cytostructural changes. At this point we can assume that dendrites are still fairly long, estradiol is bound to membrane but not nuclear receptors, and OTR and AMPAR expression is low. Lordosis behavior would be virtually nonexistent.

By 76 hours of activity, we expect that estradiol has activated nuclear receptors and stimulated genomic mechanisms, including a decrease in GluA2 and a retraction of long primary dendrites (Griffin and Flanagan-Cato, 2008), some of which may be from PAG-projecting neurons (Fig. 5.1B). These PAG-projection cells do not express ERα and undergo a decrease in spine density according to previous work in our lab (Calizo and Flanagan-Cato, 2002; Fig. 5.1C). Retraction of dendrites of PAG-projection neurons may limit the input and subsequently the output of the cells, reducing communication to the periaqueductal gray, the next nucleus involved in the lordosis circuit, as another way to prevent premature behavior. Because cells that express OTR are not involved in estradiol-induced retraction (Griffin et al., 2010) and there is a high probability that ERα is colocalized with OTR (Devidze et al., 2005), the dendrites of these supposed ERα/OTR-expressing cells remain. They also exhibit internalized OT, receive

increased synaptic innervation (Griffin et al., 2010: Ferri and Flanagan-Cato, 2012), and perhaps increased glutamate activity (Fig. 5.1A). Another subset of dendrites, short primary dendrites from non-ERα cells undergo an increase in dendritic spine density, and likely regulate local input (Calizo and Flanagan-Cato, 2000; Fig. 5.1D). The hypothesized location of AMPAR is in dendritic spines. Calcium permeability would be increased and lordosis behavior would be low. Progesterone receptors are upregulated (Boling and Blandau, 1939).

With the addition of progesterone, there is an increase in GluA1 and restoration of GluA2 to control levels, resulting in a relative increase in calcium permeability. Long primary dendrites re-extend (Griffin and Flanagan-Cato, 2008), including some PAG-projecting neurons, although spine density status on the non-PAG-projecting dendrites is not known (Calizo and Flanagan-Cato, 2002). OTR expression has extended out into the LFC (Schumacher et al., 1990), where synaptic input has decreased to control levels and glutamate activity may be diminished. Spine densities on short primary dendrites from non-ERα cells likely remain increased (Calizo and Flanagan-Cato, 2000). Lordosis is at its maximum.

In conclusion, this thesis has aimed to clarify some aspects of the microcircuitry of the vlVMH. I have 1) demonstrated that ovarian hormone-regulated neural plasticity generalizes to other species, 2) revealed neurochemically specific synaptic reorganization in the LFC, and 3) discovered early cellular mechanisms of hormone-induced spine changes. An overarching goal of this work is to elucidate how ovarian steroids act in the brain to promote sexual behavior, and how changes in wiring and chemistry may mutually contribute.

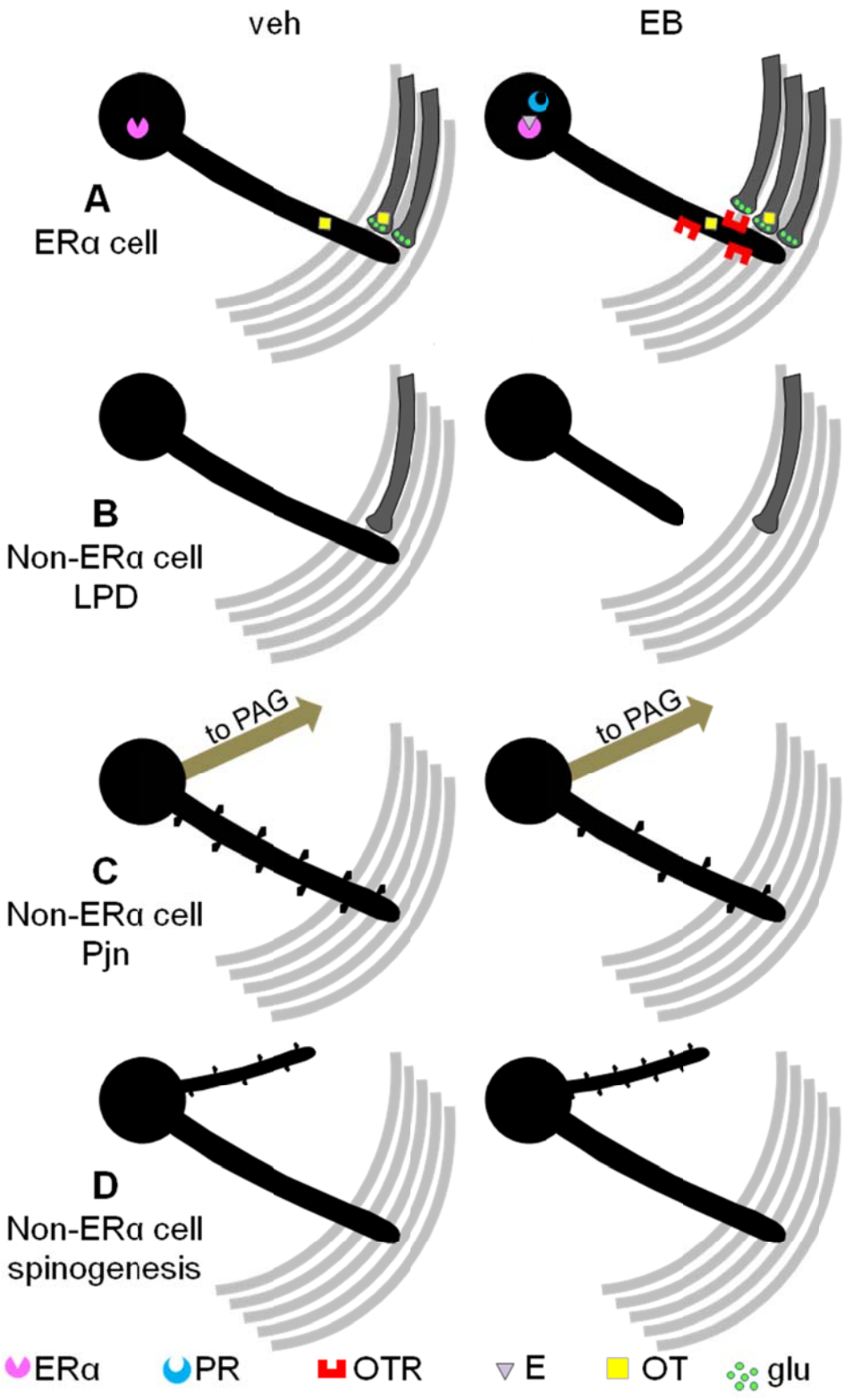

Figure 5.1 Summary of vlVMH cell types and their responses to estradiol treatment.

A. ERα-expressing cells in the vlVMH have long primary dendrites that receive more synaptic inputs from the laterl fiber complex (LFC) than non-ERα-expressing cells and contain OT. Some of the inputs colocalize OT and glutamate. In response to estradiol treatment, expression of OTR in dendrites and PR in the nucleus is increased, long primary dendrites remain extended into the LFC, and innervation.of the dendrites is further increased. B. Cells that do not express ERα receive less synaptic input than ERα-expressing cells and their dendrites undergo an estradiol-induced retraction from the LFC. C. Pjn cells do not express nuclear ERα and have axons that project to the periaqueductal gray (PAG). Estradiol decreases spine density on long primary dendrites of Pjn neurons. D. Another subset of non-ERα-expressing cells shows an estradiol-induced increase in spine density on short primary dendrites. vlVMH neurons are shown in black; gray lines represent axons in the LFC; beige arrows represent axons projecting to the PAG; ERα = estrogen receptor alpha; PR = progesterone receptor; OTR = oxytocin receptor; E = estradiol; OT = oxytocin; glu = glutamate.

APPENDIX

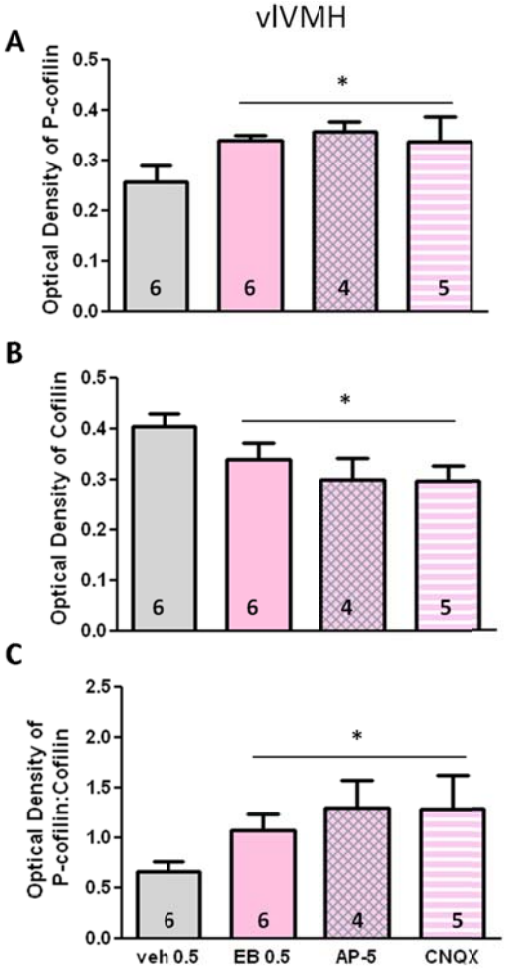

Figure A.1. The effect of AP-5 and CNQX on expression in the vlVMH.
Animals were injected icv with vehicle (veh), NMDAR antagonist AP-5, or AMPAR antagonist CNQX. Fifteen minutes later they were administered sc veh (in the veh condition only) or estradiol benzoate (EB). Animals were sacrificed 30 min later.

A. Optical density of p-cofilin. Pretreatment with AP-5 or CNQX had no effect on the estradiol-induced increase in p-cofilin. *, $p < 0.05$ veh compared to all EB-treated groups.

B. Optical density of cofilin. Pretreatment with AP-5 or CNQX had no effect on the estradiol-induced decrease in cofilin. *, $p < 0.05$ veh compared to all EB-treated groups.

C. Optical density of p-cofilin:cofilin. Pretreatment with AP-5 or CNQX had no effect on the estradiol-induced increase in p-cofilin. *, $p < 0.05$ veh compared to all EB-treated groups.

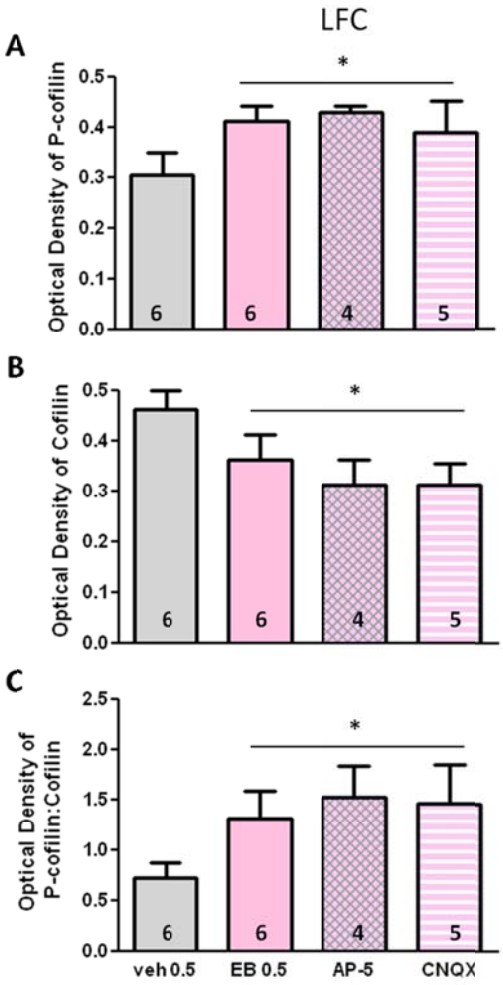

Figure A.2. The effect of AP-5 and CNQX on expression in the LFC.
Animals were injected icv with vehicle (veh), NMDAR antagonist AP-5, or AMPAR antagonist CNQX. Fifteen minutes later they were administered sc veh (in the veh condition only) or estradiol benzoate (EB). Animals were sacrificed 30 min later.

A. Optical density of p-cofilin. Pretreatment with AP-5 or CNQX had no effect on the estradiol-induced increase in p-cofilin. *, $p < 0.05$ veh compared to all EB-treated groups.

B. Optical density of cofilin. Pretreatment with AP-5 or CNQX had no effect on the estradiol-induced decrease in cofilin. *, $p < 0.05$ veh compared to all EB-treated groups.

C. Optical density of p-cofilin:cofilin. Pretreatment with AP-5 or CNQX had no effect on the estradiol-induced increase in p-cofilin. *, $p < 0.05$ veh compared to all EB-treated groups.

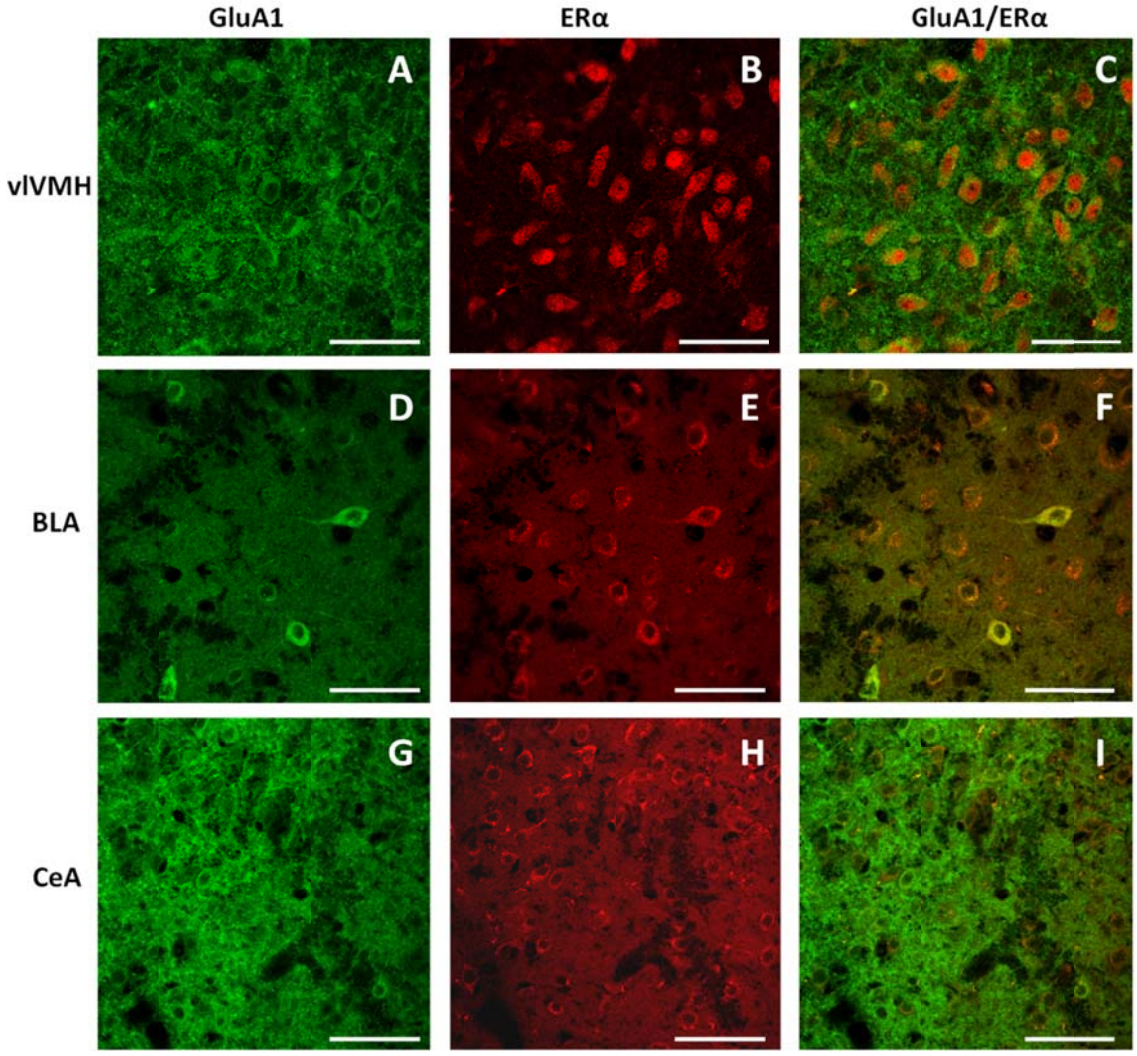

Figure A.3. Micrographs of labeling for GluA1 and ERα in the vlVMH, BLA, and CeA.
A. GluA1 labeling in the vlVMH. Dense fibers and some cytoplasmic staining are visible.
B. ERα labeling in the vlVMH. Staining is robust and nuclear.
C. GluA1/ ERα colocalization in the vlVMH. All cells that express cytoplasmic GluA1 also express nuclear ERα, but all ERα do not express cytoplasmic GluA1.
D. GluA1 labeling in the BLA. Sparse cytoplasmic staining is present.
E. ERα labeling in the BLA. Staining appears cytoplasmic.
F. GluA1/ ERα colocalization in the BLA. All cells that express cytoplasmic GluA1 also express nuclear ERα, but all ERα do not express cytoplasmic GluA1.
G. GluA1 labeling in the CeA. Dense fibers and some cytoplasmic staining are visible.
H. ERα labeling in the CeA. Abundant cytoplasmically-labeled cells are present.
I. GluA1/ ERα colocalization in the CeA. There appears to be little to no colocalization.
Bars = 50 µm.

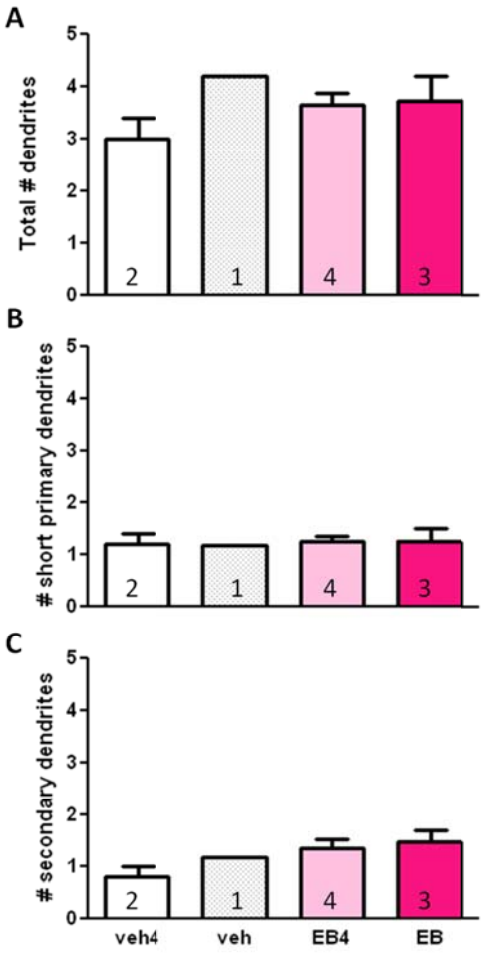

Figure A.4. Number of vlVMH dendrites.

A. Estradiol treatment did not change the total number of dendrites at four or 76 hours.

B. Estradiol treatment did not affect the number of short primary dendrites at four 76 hours.

C. Estradiol treatment did not affect the number of secondary dendrites at four 76 hours.

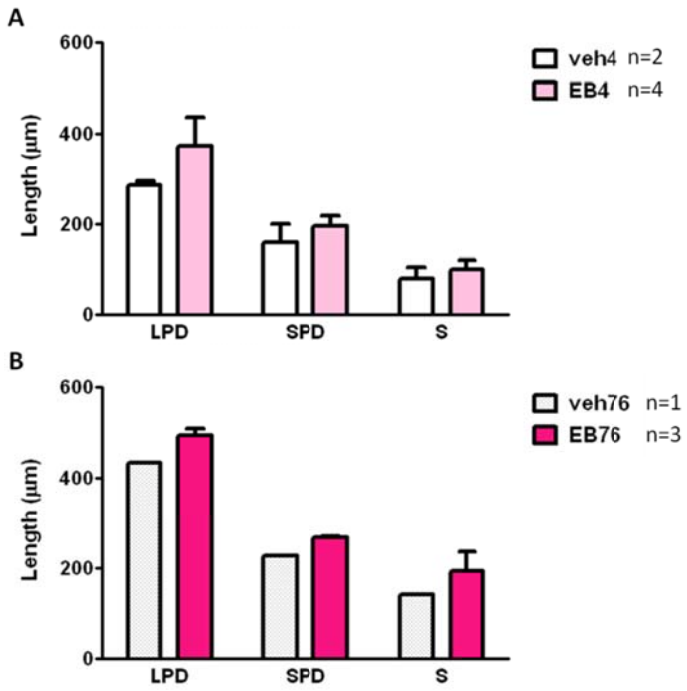

Figure A.5. Length of vlVMH dendrites.

A. Estradiol treatment did not change length of long primary dendrites (LPD), short primary dendrites (SPD), or secondary dendrites (S) at four hours.

B. Estradiol treatment did not seem to affect length of long primary dendrites (LPD), short primary dendrites (SPD), or secondary dendrites (S) at 76 hours. However, the small sample size precludes statistical analysis.

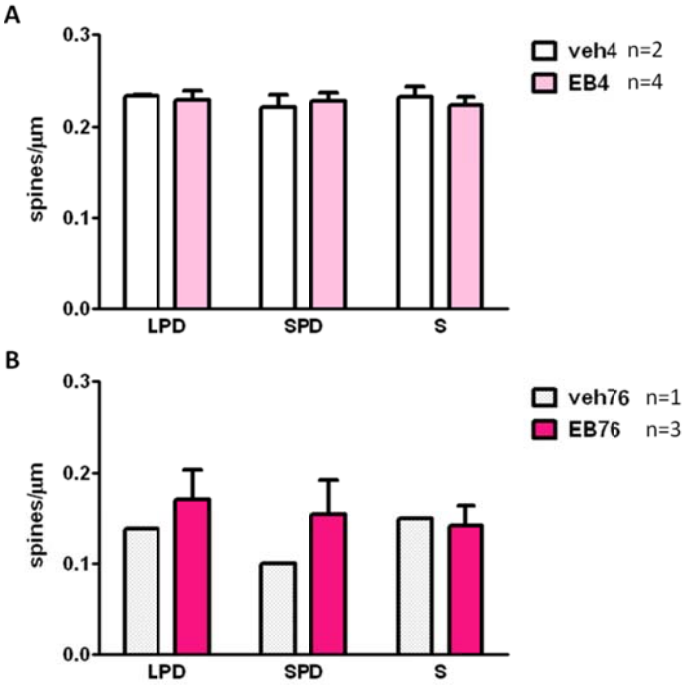

Figure A.6. Spine density of vlVMH dendrites.

A. Estradiol treatment did not change dendritic spine density on long primary dendrites (LPD), short primary dendrites (SPD), or secondary dendrites (S) at four hours.

B. Estradiol treatment did not seem to affect dendritic spine density on long primary dendrites (LPD), short primary dendrites (SPD), or secondary dendrites (S) at 76 hours. However, the small sample size precludes statistical analysis.

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